REMARKS

Claims 22-27 are presented for examination. Applicants respond below to the rejections set forth in the final Office Action and maintained by the Examiner in the Advisory Action mailed September 3, 2004.

Rejection under 35 U.S.C. § 101 – Utility

The Examiner maintains the rejection of the pending claims under 35 U.S.C. § 101 as lacking patentable utility. In the final Office Action, the Examiner acknowledged that the claimed antibodies to PRO1800 have credible utility, as there is some evidence of overexpression of PRO1800 in certain lung tumors in the data presented on page 117 (Example 16 (Gene Amplification)). Final Office Action, page 2. In the Advisory Action, however, the Examiner maintains that Applicants have not provided evidence of specific and substantial utility for PRO1800, alleging that Applicants have not provided evidence that PRO1800 is overexpressed in tumors. The Examiner asserts that the Polakis, Goddard, Grimaldi and Ashkenazi Declarations previously submitted by Applicants are fundamentally flawed. According to the Examiner, while these declarations generically state that increased mRNA levels may predict increased protein levels, they do not address overexpression of PRO1800 specifically. Further, the Examiner argues that the declarations support the position that increased levels of mRNA do not necessarily correlate with increased protein levels. According to the Examiner, evidence of overexpression of PRO1800 nucleic acids does not necessarily show that PRO1800 itself is overexpressed, and therefore the overexpression of the nucleic acids cannot serve as the foundation to support the specific utility of the claimed antibodies. The Examiner also asserts that there is no expectation that the tumors or samples used to generate the data described on page 117 of the specification are representative. Finally, the Examiner alleges that there is no diagnosis associated with overexpression of PRO1800, and that there is no evidence that PRO1800 is associated with any particular tissue at all. Thus, according to the Examiner, PRO1800 lacks specific utility.

Applicants respectfully disagree.

Utility need NOT be proven to an absolute certainty – A correlation between the evidence and the asserted utility is sufficient

Compliance with 35 U.S.C. § 101 is a question of fact. *Raytheon v. Roper*, 724 F.2d 951, 956, 220 USPQ 592, 596 (Fed. Cir. 1983) cert. denied, 469 US 835 (1984), and the evidentiary standard to be used throughout *ex parte* examination in setting forth a rejection is preponderance of the totality of the evidence under consideration. *In re Oetiker*, 977 F.2d 1443, 1445, 24 USPQ2d 1443, 1444 (Fed. Cir. 1992). Thus, to establish a *prima facie* case of lack of utility, the Examiner **must establish that it is more likely than not that one of ordinary skill in the art would doubt the truth of Applicants' statement of utility.** *Id.* If the PTO meets its burden of setting forth sufficient evidence to establish a *prima facie* case a lack of utility, only then does the burden shift to the Applicant to provide rebuttal evidence. *In re Brana*, 51 F.3d 1560, 1566, 34 U.S.P.Q.2d 1436 (Fed. Cir. 1995). Such rebuttal evidence does not need to absolutely prove that the asserted utility is real. Rather, the evidence only needs to be reasonably indicative of the asserted utility. See M.P.E.P. at § 2107.02, part VII (2004).

In *Fujikawa v. Wattanasin*, 93 F.3d 1559, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996), the Court of Appeals for the Federal Circuit upheld a PTO decision that *in vitro* testing of a novel pharmaceutical compound was sufficient to establish practical utility, stating the following rule:

> [T]esting is often required to establish practical utility. But the test results **need not absolutely prove** that the compound is pharmacologically active. All that is required is that the tests be "*reasonably* indicative of the desired [pharmacological] response." In other words, there must be **a sufficient correlation** between the tests and an asserted pharmacological activity so as to convince those skilled in the art, **to a reasonable probability**, that the novel compound will exhibit the asserted pharmacological behavior." *Fujikawa v. Wattanasin*, 93 F.3d 1559, 1564, 39 U.S.P.Q.2d 1895 (Fed. Cir. 1996) (internal citations omitted, bold emphasis added, italics in original).

While the *Fujikawa* case was in the context of utility for pharmaceutical compounds, the principles stated by the Court are applicable in the instant case where the asserted utility is for a diagnostic use – utility does not have to be established to an absolute certainty, rather, the evidence must convince a person of skill in the art "to a reasonable probability." In addition, the evidence need not be direct, so long as there is a "sufficient correlation" between the tests performed and the asserted utility.

The Court in *Fujikawa* relied in part on its decision in *Cross v. Iizuka*, 753 F.2d 1040, 224 U.S.P.Q. 739 (Fed. Cir. 1985). In *Cross*, the Appellant argued that basic *in vitro* tests conducted in cellular fractions did not establish a practical utility for the claimed compounds. Appellant argued that more sophisticated *in vitro* tests using intact cells, or *in vivo* tests, were necessary to establish a practical utility. The Court in *Cross* rejected this argument, instead favoring the argument of the Appellee:

> *[I]n vitro* results...are generally predictive of *in vivo* test results, i.e., there is a **reasonable correlation** therebetween. Were this not so, the testing procedures of the pharmaceutical industry would not be as they are. [Appellee] has not urged, and rightly so, that there is an invariable exact correlation between *in vitro* test results and *in vivo* test results. Rather, [Appellee's] position is that successful *in vitro* testing for a particular pharmacological activity establishes a **significant probability** that *in vivo* testing for this particular pharmacological activity will be successful. *Cross v. Iizuka*, 753 F.2d 1040, 1050, 224 U.S.P.Q. 739 (Fed. Cir. 1985) (emphasis added).

Fujikawa and *Cross* establish that the legal standard for demonstrating utility is a relatively low hurdle. An Applicant need only provide evidence such that it is **more likely than not that a person of skill in the art would be convinced, to a reasonable probability, that the asserted utility is true**. Under the holding in *Cross*, Applicants need not establish that amplification of the PRO1800 gene necessarily results in PRO1800 polypeptide overexpression in order to satisfy the requirements under 35 U.S.C. § 101 for utility. Rather, Applicants need only establish that given the gene amplification data set forth in Example 16, there is a significant probability that the overexpression of PRO1800 polypeptides tracks gene amplification in colon and lung tumors.

Even assuming that the PTO has met its initial burden to offer evidence that one of ordinary skill in the art would reasonably doubt the truth of the asserted utility, which Applicants maintain is not the case, Applicants assert that they have met their burden of providing rebuttal evidence such that it is more likely than not those skilled in the art, to a reasonable probability, would believe that the PRO1800 polypeptide is useful as a diagnostic tool for cancer.

PRO1800 has a specific and substantial utility in the diagnosis and treatment of colon and lung tumors

Applicants submit that the evidence of record establishes a specific and substantial utility for PRO1800 polypeptides in the diagnosis and treatment of cancer. In particular, Applicants

have submitted the data provided in Example 16 of the instant specification showing that the gene encoding PRO1800 is amplified at least two-fold in certain colon and lung tumors. The specification states that "[a]mplification is associated with overexpression of the gene product, indicating that the polypeptides are useful targets for therapeutic intervention in certain cancers. . .and diagnostic determination of the presence of those cancers."

Having asserted substantial and specific utility for PRO1800, the burden is thus on the PTO to establish that it is more likely than not that one of skill in the art would doubt the truth of the asserted utilities, i.e., it is more likely than not that one of skill in the art would doubt that PRO1800 is overexpressed based on the gene amplification data. Applicants respectfully submit that the PTO has failed to provide evidence showing that it is *more likely than not* one of skill in the art would doubt that PRO1800 levels correlate with gene amplification.

The Examiner relies on Konopka *et al.* (PNAS 83:4049-4052 (1986)) and Pennica *et al.* (PNAS, 95:14717-14722 (1998)), for the proposition that there is no *necessary* relationship between nucleic acid expression in a cell and protein expression. As Applicants have pointed out, these references do not support the PTO's position. First, the Examiner alleges that Konopka's statement "[p]rotein expression is not related to amplification of the *abl* gene but to variation in the level of *bcr-abl* mRNA produced from a single Phl template" shows that gene amplification and protein overexpression are not necessarily correlated. In fact, the *bcr-abl* mRNA levels described in Konopka arose from the result of a chromosomal translocation. Thus, Konopka merely shows that gene amplification is not the *only* way by which proteins can be overexpressed, a fact long recognized in the art. See, e.g., Benjamin Lewin, Genes V, 5th ed. 1994, pages 1196-1201, submitted herewith as Exhibit 1. Pennica *et al.* also does not support the Examiner's proposition that it is more likely than not that gene amplification and protein overexpression are not correlated. Although Pennica reported apparent amplification of the *WISP-2* gene without a concomitant increase in mRNA levels, the authors took care to point out that the "apparent amplification" of the gene encoding *WISP-2* may have been an artifact. (Pennica at 14722). In sum, the two references relied upon by the Examiner in the Final Office Action do not provide strong, if any, support for a *prima facie* showing of lack of utility.

Those of skill in the art believe that a correlation more likely than not exists between levels of gene amplification and corresponding proteins

Applicants submit that specific and substantial utility for PRO1800 polypeptides is established by showing that those of skill in the art recognize that there is a general correlation between gene amplification and altered protein levels. Specifically, those of skill in the art would not doubt that the PRO1800 polypeptides are overexpressed in colon and lung tumors and are therefore useful diagnostic tools and therapeutic targets as described in the specification. As discussed above, the evidence of utility does not need to be a *necessary* connection between PRO1800 gene amplification and PRO1800 protein expression. Rather, Applicants need only establish that it is more likely than not that a person of skill in the art would be convinced, to a reasonable probability, that PRO1800 polypeptides and antibodies that bind thereto are useful as targets for therapeutics and as diagnostic tools.

The teachings in Genes V, a leading textbook in the field, illustrate that at the time the instant application was filed, it was well known by those of skill in the art that gene amplification leads to overexpression of the corresponding gene product. Benjamin Lewin, Genes V, 5th ed. 1994, pages 1196-1201, submitted herewith as Exhibit 1. In a section entitled "Insertion, translocation, or amplification may activate proto-oncogenes", the text describes various molecular events that lead to overexpression of a gene product, using the *c-myc* gene as an example. The first mechanism taught is insertion of a retrovirus upstream of the gene which causes it to be driven by a more efficient promoter, resulting in increased mRNA and protein levels. Next, Lewin teaches that chromosomal translocations may bring a gene to a new region where it is actively expressed, resulting in increased gene and protein expression. The third mechanism whereby protein levels of oncogenes are overexpressed is gene amplification. The text emphasizes that the common thread among the different means of activation of proto-oncogenes is that the expression of the gene is increased. Thus, as of 1994, it was well-known in the art that gene amplification is correlated with overexpression of the corresponding mRNA and encoded protein.

Additional information regarding the understanding of those of skill in the art regarding the relationship between gene amplification and protein overexpression at the time the instant application was filed is found in Alitalo (Med. Biol.,. 62:304-317 (1984), submitted herewith as Exhibit 2), and Merlino *et al.* (J. Clin. Invest., 75:1077-1079 (1985), submitted herewith as

Exhibit 3). Alitalo teaches gene amplification of oncogenes results in elevated expression of the gene, and that the increased dosage of the gene product may contribute to the progression of some cancers. Merlino *et al.* studied epidermoid carcinoma cells, and teach that amplification of the EGF receptor gene results in increased levels of EGF receptor mRNA and increased levels of EGF receptor protein. Taken together, the excerpt from Genes V, as well as the Alitalo and Merlino references, establish that as of the filing date of the instant application, those of skill in the art appreciated the correlation between gene amplification and overexpression of the encoded gene product.

The teachings of Genes V, Alitalo, and Merlino are confirmed in several more recent reports that also document the correlation between gene amplification and levels of protein. Applicants submit herewith two more recent studies providing evidence that the teachings referred to above are still widely accepted by those of skill in the art. Bahnassy *et al.* (BMC Gastroenterology, 4:22-34 (2004), submitted herewith as Exhibit 4) studied the amplification of *cyclin D1, cyclin A, histone H3* and *Ki-67*, and assessed the levels of the encoded proteins by immunohistochemistry. Bahnassy *et al.* found a "significant correlation between *cyclin D1* gene amplification and protein overexpression" (Bahnassy at 27, column 1). Similarly, Blancato *et al.* (British Journal of Cancer, 90(8), 1612-1619 (2004), submitted herewith as Exhibit 5), report that overexpression of *c-myc* mRNA and c-Myc protein is related to the copy number of the *c-myc* amplification (Blancato at 1613, column 2). Bahnassy and Blancato demonstrate continued evidentiary support for the widely-accepted principle that gene amplification correlates with overexpression of the encoded protein.

Several references also document the correlation between gene copy number and mRNA levels. The teachings in Molecular Biology of the Cell, a leading textbook in the field (Bruce Alberts, *et al.*, Molecular Biology of the Cell (4th ed. 2002) submitted herewith as Exhibit 6), support the previously submitted testimony of Polakis and Grimaldi regarding the correlation between mRNA levels and protein levels. Figure 6-3 on page 302 illustrates the basic principle that there is a correlation between increased gene expression and increased protein expression. The accompanying text states that "a cell can change (or regulate) the expression of each of its genes according to the needs of the moment – *most obviously by controlling the production of its mRNA*." Id. at 302, emphasis added. Similarly, figure 6-90 on page 364 illustrates the path from gene to protein. The accompanying text states that while potentially each step can be regulated

by the cell, "**the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes**." Id. at 364. This point is repeated on page 379, where the authors state that of all the possible points for regulating protein expression, "**[f]or most genes transcriptional controls are paramount**." Id. at 379. Thus, in light of the general understanding that mRNA levels typically correlate with protein levels, the several references correlating gene amplification with increased mRNA levels provide strong evidence that the levels of PRO1800 polypeptides tracks the gene copy number. For example, Ornoft *et al.* (Molecular and Cellular Proteomics, 1:37-45 (2002), submitted herewith as Exhibit 7) studied transcript levels of 5600 genes in malignant bladder cancers which were linked to a gain/loss of chromosomal material using an array-based method. Ornoft *et al.* found "in general (18 of 23 cases) chromosomal areas with more than 2-fold gain of DNA showed a corresponding increase in mRNA transcripts" (Ornoft at 37, column 1, abstract). Hyman *et al.* (Cancer Research, 62:6240-6245 (2002), submitted herewith as Exhibit 8) used CGH analysis and microarrays to compare DNA copy numbers and mRNA expression of over 12, 000 genes in breast cancer tumors and cell lines. They showed that there is "evidence of a prominent global influence of copy number changes on gene expression levels" (Hyman at 6244, column 1, last paragraph). Pollack *et al.* (PNAS, 99:12963-12968 (2002), submitted herewith as Exhibit 9) studied a series of primary human breast tumors. Pollack *et al.* report "changes in DNA copy number have a large, pervasive, direct effect on global gene expression patterns in both breast cancer cell lines and tumors." (Pollack at 12967, column 1). Meric *et al.* (Molecular Cancer Therapeutics, 1:971-979 (2002), submitted herewith as Exhibit 10), teaches:

> The **fundamental principle** of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells...[M]ost efforts have concentrated on identifying differences in gene expression at the level of mRNA, which can be attributable to either DNA amplification or to differences in transcription. Meric et al. at 971 (emphasis added).

Together, the previously submitted declarations with their accompanying references, the excerpt from Genes V, and the references discussed above, establish that the accepted understanding in the art is that there is a *reasonable* correlation between gene amplification and the level of the corresponding mRNA and protein. As the Examiner correctly points out, while an increase in gene copy number does not necessarily correlate with an increase in protein

expression, Applicants respectfully submit that the Examiner is requiring Applicants to establish to a certainty that PRO1800 is also overexpressed, rather than whether one of ordinary skill in the art would more likely than not believe that PRO1800 is overexpressed in colon and lung tumors showing amplification of the gene. Applicants have provided sufficient evidence to establish that it is more likely than not that one of skill in the art would believe that, because the gene encoding PRO1800 is amplified at least two-fold in several colon tumors and lung tumors, the PRO1800 polypeptide is expressed at a higher level in these tumors. Thus, in light of all the evidence on the record, Applicants submit that one of skill in the art is more likely than not to believe Applicants' asserted utilities for PRO1800.

The claimed antibodies would have diagnostic utility even if there is no direct correlation between gene amplification and protein expression

The evidence on the record establishes that it is more likely than not that one of skill in the art would believe that there is a correlation between gene amplification and overexpression of the gene and gene product. However, even assuming *arguendo* that this was not the case, the claimed antibodies would still have specific and substantial utility.

As explained in paragraph 6 of the previously submitted Ashkenazi Declaration:

> Even when amplification of a cancer marker gene does not result in significant over-expression of the corresponding gene product, this very absence of gene product over-expression still provides significant information for cancer diagnosis and treatment. Thus, if over-expression of the gene product does not parallel gene amplification in certain tumor types but does so in others, then parallel monitoring of gene amplification and gene product over-expression enables more accurate tumor classification and hence better determination of suitable therapy. In addition, absence of over-expression is crucial information for the practicing clinician. If a gene is amplified but the corresponding gene product is not over-expressed, the clinician accordingly will decide not to treat a patient with agents that target that gene product.

This statement is echoed by Grimaldi in his previously submitted declaration at paragraph 6, and is further supported by the teachings in the article by Hanna and Mornin (Pathology Associates Medical Laboratories, (1999), submitted herewith as Exhibit 11). The article teaches that the HER-2/neu gene has been shown to be amplified and/or overexpressed in 10%-30% of invasive breast cancers and in 40-60% of intraductal breast carcinoma. Further, the article teaches that diagnosis of breast cancer includes testing both the amplification of the HER-2/neu gene (by FISH) as well as the overexpression of the HER-2/neu gene product (by IHC). Even

when the protein is not overexpressed, the assay relying on both tests leads to a more accurate classification of the cancer and a more effective treatment of it. Press *et al.* (*J. Clin. Oncol.*, 20:3095-3115, (2002), submitted herewith as Exhibit 12). Press stresses "the importance of HER2/neu in breast cancer treatment decision making has focused attention on the ability of various clinical assays to correctly assign HER-2/neu amplification and expression status. (Press at 3095, Col. 1). Press teaches that measurement of gene amplification by fluorescent *in situ* hybridization (FISH) accompanied by immunohistochemical assays with anti-HER-2 antibodies is desirable in determining whether antibody therapy is appropriate for the treatment of breast cancer. Thus, the evidence of record establishes another utility for PRO1800 polypeptides based on the gene amplification data.

Applicants have established that it is the general, accepted understanding in the art that gene amplification leads to increased gene and protein expression. Because this is the general rule, even when this is not the case, a polypeptide encoded by a gene that is amplified in tumors would still have utility. Applicants have provided two references establishing the utility of measuring the gene product of an amplified gene. These references submitted herewith demonstrate a "reasonable" confirmation of the asserted utility for the claimed antibodies.

In view of the foregoing, Applicants respectfully request that the Examiner reconsider and withdraw the rejections under 35 U.S.C. § 101.

Conclusion

Applicants have established that it is the general, accepted understanding in the art that gene amplification leads to increased gene and protein expression. Of particular significance is the fact that the statements in the Genes V excerpt, as well as the general statements in the references submitted herein, have identified the general understanding in the field regarding the correlation between gene amplification and overexpression of the gene and gene product. The references relied upon by the Examiner do not weaken Applicants' showing. Applicants respectfully submit that the totality of the above-cited evidence clearly establishes that those of skill in the art would believe that amplification of the gene more likely than not correlates with increased protein levels. In light of the fact that Applicants need not show a *necessary* correlation between gene amplification and protein levels, Applicants respectfully submit that they have rebutted any prima facie case of non-utility and non-enablement the Examiner may have established. Accordingly, Applicants request withdrawal of the rejection of Claims 22-27.

Rejection under 35 U.S.C. § 112, first paragraph – Enablement

The Examiner maintains the rejection of Claims 22-27 under 35 U.S.C. § 112, first paragraph, as not being enabled by the specification. Specifically, the Examiner asserts that since PRO1800 has no utility, the claimed antibodies are not enabled.

For the reasons set forth above, Applicants submit that PRO1800 and antibodies that bind PRO1800 have credible, substantial, and specific utility. Thus, Applicants respectfully request that the Examiner reconsider and withdraw the rejections under 35 U.S.C. § 112, first paragraph.

CONCLUSION

In view of the above, Applicants respectfully maintain that the claims are patentable and request that they be passed to issue. Applicants invite the Examiner to call the undersigned if any remaining issues may be resolved by telephone.

Please charge any additional fees, including any fees for additional extension of time, or credit overpayment to Deposit Account No. 11-1410.

Respectfully submitted,

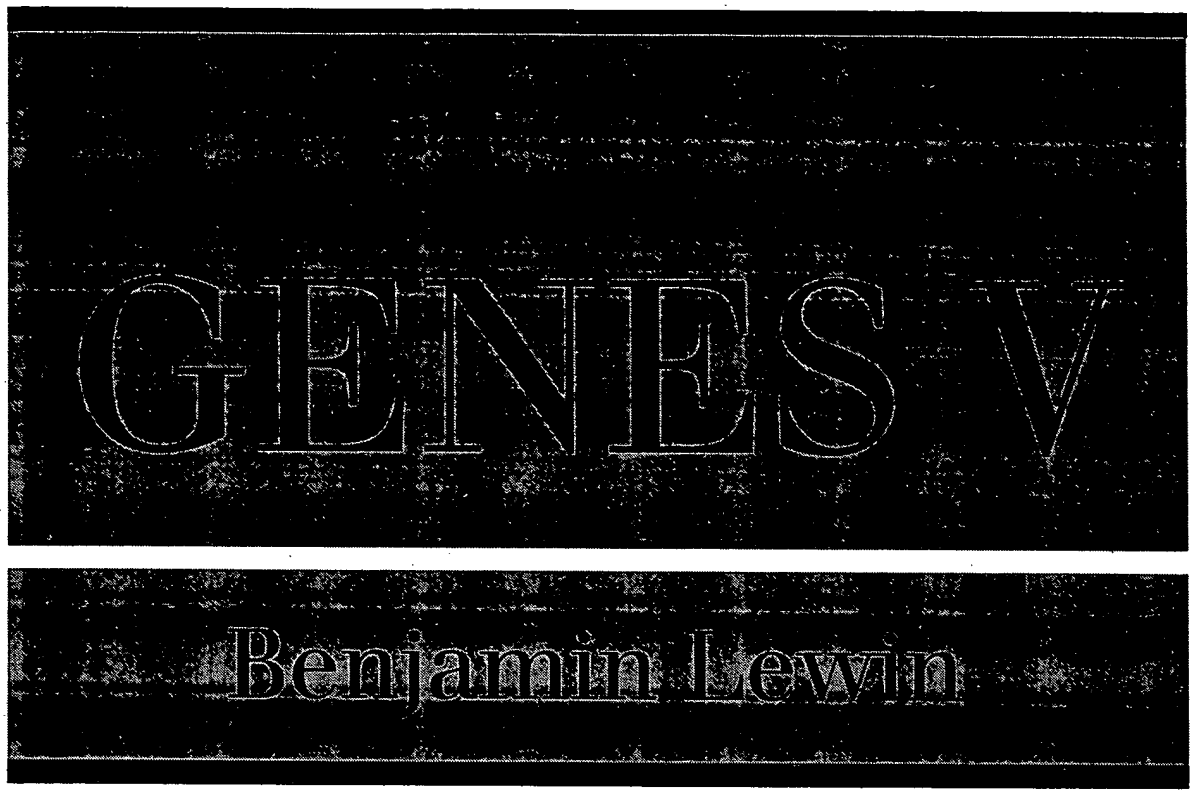
KNOBBE, MARTENS, OLSON & BEAR, LLP

Dated: Dec. 22, 2004

By: _____

AnneMarie Kaiser
Registration No. 37,649
Attorney of Record
Customer No. 30,313
(619) 235-8550

S:\DOCS\KXM\KXM-1161.DOC
121704



OXFORD UNIVERSITY PRESS
Oxford New York Tokyo
1994

~10×). Also, some tumor lines have amplified *ras* genes. A 20-fold increase in the level of a nontransforming Ras protein is sufficient to allow the transformation of some cells. The effect has not been fully quantitated, but it suggests the general conclusion that oncogenesis depends on overactivity of Ras protein, and is caused either by increasing the amount of protein or (probably more efficiently) by a variety of mutations that increase the activity of the protein.

Transfection by DNA can be used to transform only certain cell types. Although transforming oncogenes have been isolated from both rodent and human cells, most targets for transformation by transfection with oncogenes have been rodent fibroblasts in culture. (In fact, the difference in the source of the oncogene [human] and the recipient cell [rodent] is an important factor in allowing the donor gene to be distinguished unequivocally from recipient DNA.) Limitations of the assay explain why relatively few oncogenes have been detected by transfection. This system has been most effective with *ras* genes, where there is extensive correlation between mutations that activate *c-ras* genes in transfection and the occurrence of tumors.

Insertion, translocation, or amplification may activate proto-oncogenes

A variety of genomic changes can activate proto-oncogenes, sometimes involving a change in the target gene itself, sometimes activating it without changing the protein product. In cases of insertion and translocation, there is evidence that the genomic change is the causative event; in cases of amplification there is a correlation with tumorigenesis, but no direct proof for a causative role.

Many tumor cell lines have visible regions of chromosomal amplification, as shown by homogeneously staining regions (see Figure 36.27) or double minute chromosomes (see Figure 36.28). In some cases, the amplified region contains a known oncogene or a gene related to one. In other cases, where amplification is not visible, the use of batteries of probes representing oncogenes shows that a particular oncogene is amplified. Examples of oncogenes that are amplified in various tumors include *c-myc, c-abl, c-myb, c-erbB, and c-K-ras.*

Established cell lines are prone to amplify genes (it is one of several karyotypic changes to which they are susceptible). All the same, the presence of known oncogenes in the amplified regions, and the consistent amplification of particular oncogenes in many independent tumors of the same type, again strengthens the correlation between increased expression and tumor growth. Of course, it is possible that the gene amplification gives an advantage to growth of the established tumor; it is not necessarily an event involved in its initiation.

Some proto-oncogenes are activated by events that change their expression, but which leave their coding sequence unaltered. The best characterized is *c-myc,* whose expression is elevated by several mechanisms. One common mechanism is the insertion of a nondefective retrovirus in the vicinity of the gene.

The ability of a retrovirus to transform without expressing a *v-onc* sequence was first noted during analysis of the bursal lymphomas caused by the transformation of B lymphocytes with avian virus. Similar events occur in the induction of T cell lymphomas by murine leukemia virus. In each case, the transforming potential of the retrovirus seems to lie with its LTR rather than with a coding sequence.

In many independent tumors, the virus has integrated into the cellular genome within or close to the *c-myc* gene. The gene consists of three exons; the first represents a long nontranslated leader, and the second two code for the c-Myc

protein. Figure 39.8 summarizes the types of insertion at this locus. The retrovirus may be inserted at a variety of locations relative to the *c-myc* gene.

The simplest insertions to explain are those that occur within the first intron. The LTR provides a promoter, and transcription reads through the two coding exons. Transcription of *c-myc* under viral control differs in two ways from its usual expression: the level of expression is increased (because the LTR provides an efficient promoter); and the transcript lacks its usual nontranslated leader (which may usually limit expression).

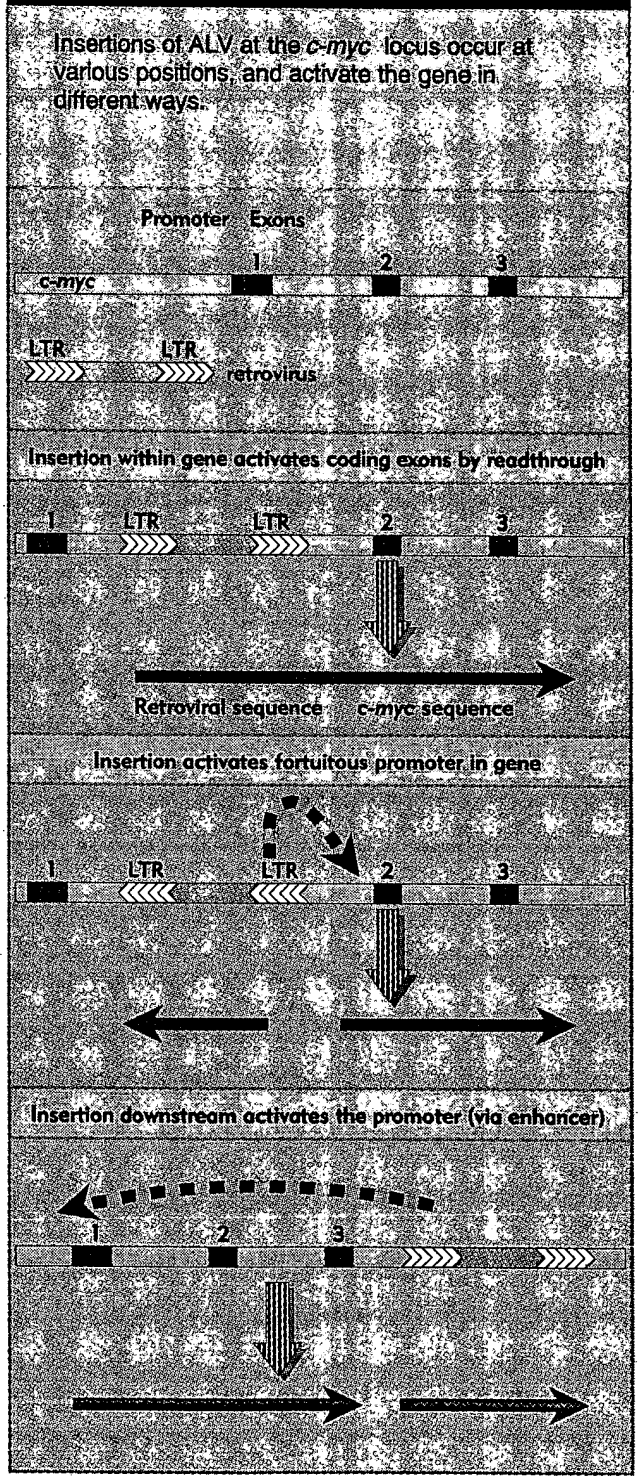
Activation of *c-myc* in the other two classes of insertions reflects different mechanisms. The retroviral genome may be inserted within or upstream of the first intron, but in reverse orientation, so that its promoter points in the wrong direction. Probably the LTR provides an enhancer that acts on an upstream sequence that fortuitously resembles a promoter. The retroviral genome also may be inserted downstream of the *c-myc* gene, in which case transcription initiates at the usual *c-myc* promoter(s), but is increased by the enhancer in the retroviral LTR.

In all of these cases, the coding sequence of c-myc *is unchanged, so oncogenicity is attributed to the loss of normal control, and increased expression, of the gene.*

Other oncogenes that are activated in tumors by the insertion of a retroviral genome include *c-erbB, c-myb, c-mos, c-H-ras,* and *c-raf.* Up to 10 other cellular genes (not previously identified as oncogenes by their presence in transforming viruses) are implicated as potential oncogenes by this criterion. The best characterized among this latter class are *wnt1* and *int2*. The *wnt1* gene codes for a protein involved in early embryogenesis that is related to the *wingless* gene of *Drosophila; int2* codes for a growth factor related to FGF.

Translocation to a new chromosomal location is another of the mechanisms by which oncogenes are activated. Certain chromosomal translocations are consistently associated with activation of oncogenes that lie near the breakpoints. This situation was originally discovered via a connection between the loci coding immunoglobulins and the

Figure 39.8



occurrence of certain tumors. Specific chromosomal translocations are often associated with plasmacytomas in the mouse and with Burkitt lymphomas in man. These tumors arise from undifferentiated B lymphocytes. The common feature in both species is that an oncogene on one chromosome is brought into the proximity of an Ig locus on another chromosome. Similar events occur in T lymphocytes to bring oncogenes into the proximity of a TcR locus.

The basic cause of the translocation event is a malfunction of the system responsible for recombining V and C genes or switching between IgH C genes. Instead of acting on two sites within the Ig or TcR locus, the system recombines the immune locus with an unrelated region on a different chromosome. This results in a **reciprocal translocation**, which is illustrated in **Figure 39.9**.

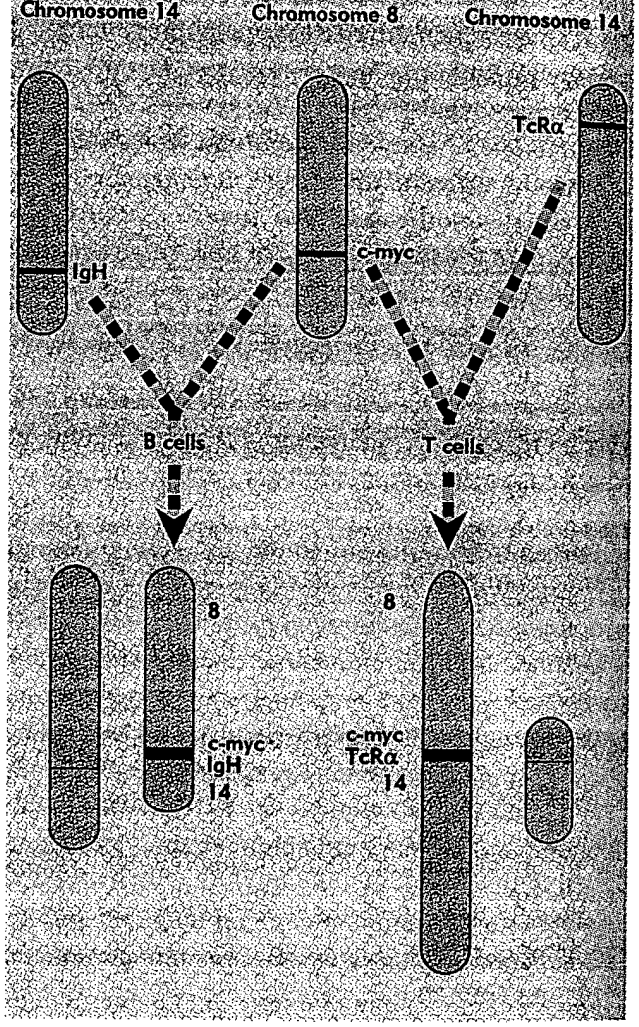
We do not know the basis for the involvement of the nonimmune partner, but in both man and mouse it is often the *c-myc* locus. In man, the translocations in B cell tumors usually involve chromosome 8, which carries *c-myc,* and chromosome 14, which carries the IgH locus; ~10% involve chromosome 8 and either chromosome 2 (κ locus) or chromosome 22 (lambda locus). The translocations in T cell tumors often involve chromosome 8, and either chromosome 14 (which has the TcRα locus at the other end from the Ig locus) or chromosome 7 (which carries TcR β locus). Analogous translocations occur in the mouse.

Translocations at the IgH locus in B cells fall into two classes. One type is similar to those observed at other Ig loci and at TcR loci, involving the consensus sequences used for V-D-J somatic recombination of active Ig genes. In the other type, the translocation occurs at a switching site, so these cases may be associated with function of the system that switches expression from one C_H gene to another.

When *c-myc* is translocated to the Ig locus, its level of expression is usually increased. The increase varies considerably among individual tumors, generally being in the range from 2 to 10. Why does translocation activate the *c-myc* gene? The translocation event does not involve fixed sites,

Figure 39.9

A chromosomal translocation is a reciprocal event that exchanges parts of two chromosomes. Translocations that activate the human *c-myc* proto-oncogene involve Ig loci in B cells and TcR loci in T cells.



but occurs at a variety of locations within a general region on each recombining chromosome. The event has two consequences: *c-myc* is brought into a new region, one in which an Ig or TcR gene was actively expressed; and the structure of the *c-myc* gene may itself be changed (but usually not involving the coding regions). It seems likely that several different mechanisms can activate the *c-myc* gene

in its new location (just as retroviral insertions activate *c-myc* in a variety of ways).

The correlation between the tumorigenic phenotype and the activation of *c-myc* by either insertion or translocation suggests that continued high expression of the c-Myc protein is oncogenic. Expression of *c-myc* must be switched off to enable immature lymphocytes to differentiate into mature B and T cells; failure to turn off *c-myc* maintains the cells in the undifferentiated (dividing) state. The oncogenic potential of *c-myc* has been demonstrated directly by the creation of transgenic mice carrying a normal *c-myc* gene linked to an enhancer. Transgenic mice carrying a *c-myc* gene linked to a B lymphocyte-specific enhancer (the IgH enhancer) develop lymphomas. The tumors represent both immature and mature B lymphocytes, suggesting that over-expression of *c-myc* is tumorigenic throughout the B cell lineage. Transgenic mice carrying a *c-myc* gene under the control of the LTR from a mouse mammary tumor virus, however, develop a variety of cancers, including mammary carcinomas. This suggests that increased or continued expression of *c-myc* transforms the type of cell in which it occurs into a corresponding tumor. Specificity of the tumor type may therefore depend on the mechanism used to activate *c-myc*; it is not an intrinsic property of the gene.

c-myc exhibits three means of oncogene activation: retroviral insertion, chromosomal translocation, and gene amplification. The common thread among them is increased expression of the oncogene rather than a qualitative change in its coding function, although in at least some cases the transcript has lost the usual (and possibly regulatory) nontranslated leader. *c-myc* provides the paradigm for oncogenes that may be effectively activated by increased (or possibly altered) expression.

Every translocation generates reciprocal products; sometimes a known oncogene is activated in one of the products, but in other cases it is not evident which of the reciprocal products has responsibility for oncogenicity. Also, it is not axiomatic that the gene(s) at the breakpoint have responsibility; for example, the translocation could provide an enhancer that activates another gene nearby.

A variety of translocations found in B and T cells have identified new oncogenes. In some cases, the translocation generates a hybrid gene, in which an active transcription unit is broken by the translocation. This has the result that the exons of one gene may be connected to another. In such cases, there are two potential causes of oncogenicity: the proto-oncogene part of the protein may be activated in some way that is independent of the other part, for example, because it is over-expressed under its new management (a situation directly comparable with the example of *c-myc*); or the other partner in the hybrid gene may have some positive effect that generates a gain-of-function in the part of the protein coded by the proto-oncogene.

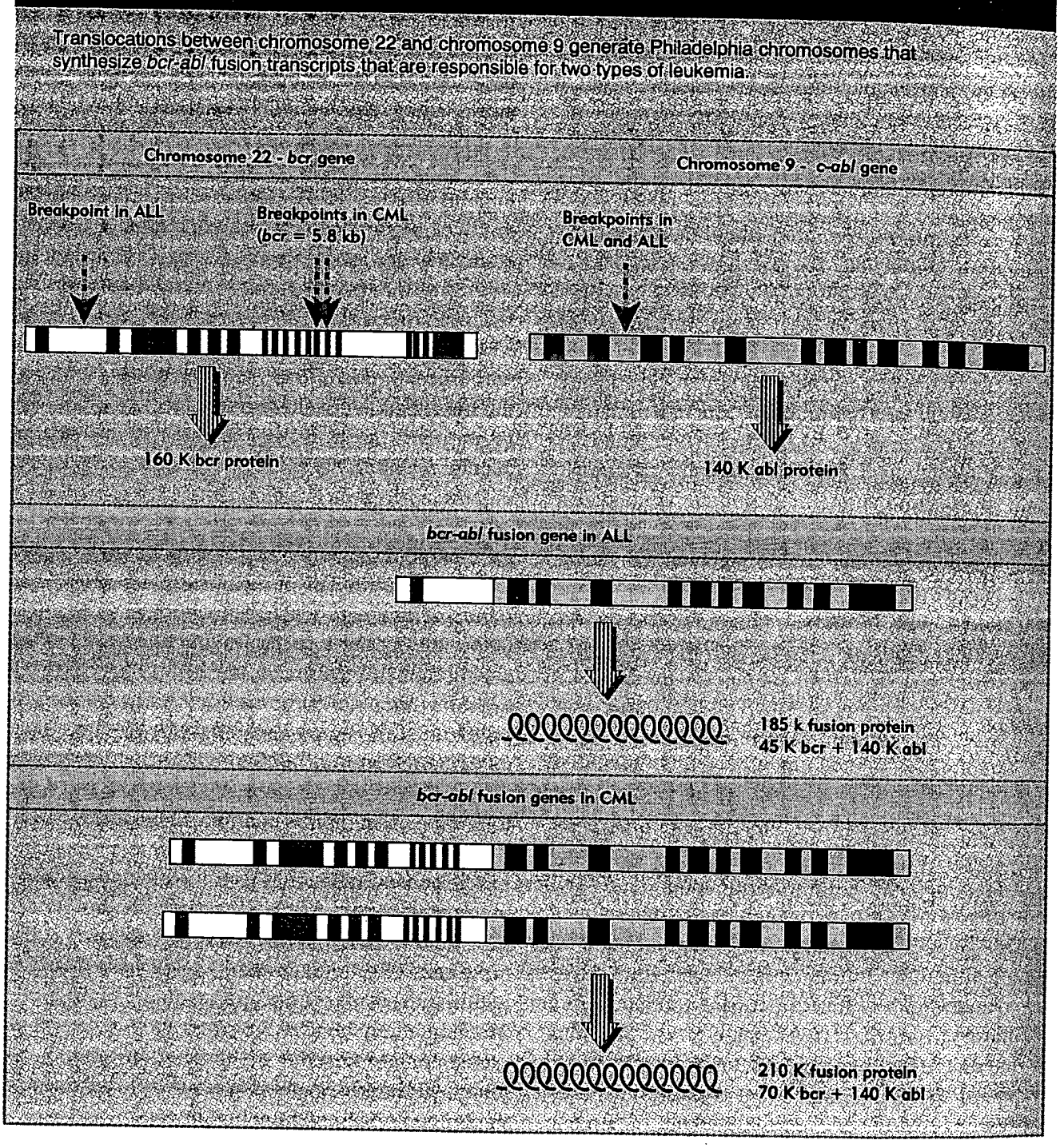
One of the best characterized cases in which a translocation creates a hybrid oncogene is provided by the *Philadelphia (PH¹)* chromosome present in patients with chronic myelogenous leukemia (CML). This reciprocal translocation is too small to be visible in the karyotype, but links a 5,000 kb region from the end of chromosome 9 carrying *c-abl* to the *bcr* region of chromosome 22. The *bcr* (*b*reak-point *c*luster *r*egion) was originally named to describe a region of ~5.8 kb within which breakpoints occur on chromosome 22. Different cases of CML have breakpoints at different locations within this region.

The consequences of this translocation are summarized in **Figure 39.10**. The *bcr* region lies within a large (>90 kb) gene, which is now known as the *bcr* gene. The breakpoints in CML usually occur within one of two introns in the middle of the gene. The same gene is also involved in translocations that generate another disease, ALL (acute lymphoblastic leukemia); in this case, the breakpoint in the *bcr* gene occurs in the first intron.

The *c-abl* gene is expressed by alternative splicing that uses either of the first two exons. The breakpoints in both CML and ALL occur in the intron that precedes the first common exon. Although the exact breakpoints on both chromosomes 9 and 22 vary in individual cases, the

Figure 39.10

Translocations between chromosome 22 and chromosome 9 generate Philadelphia chromosomes that synthesize *bcr-abl* fusion transcripts that are responsible for two types of leukemia.



common outcome is the production of a transcript coding for a Bcr–Abl fusion protein, in which N-terminal sequences derived from *bcr* are linked to *c-abl* sequences. In ALL, the 185,000 dalton fusion protein has ~45,000 daltons of the Bcr protein linked to c-Abl; in CML the fusion protein of 210,000 daltons has ~70,000 daltons of the Bcr protein. In each case, the fusion protein contains ~140,000

daltons of the usual ~145,000 c-Abl protein, that is, it has lost just a few N-terminal amino acids of the c-Abl sequence.

Why is the fusion protein oncogenic? It relies on an interaction between the N-terminal region provided by *bcr* with the c-Abl protein. The *bcr* gene has a variety of sequence motifs related to proteins involved in signalling pathways, but the pertinent one is a serine/threonine kinase activity that is coded within the first exon. This autophosphorylates residues in this part of the protein, and the phosphorylation confers the ability to interact with a region of the c-Abl protein called the SH2 domain (we discuss the nature of SH2 domains later). This raises the possibility that the Bcr part of the protein interacts with the c-Abl sequences, perhaps changing their conformation and activating a latent oncogenic potential.

Changes at the N-terminus are involved in the activation of oncogenic activity of *v-abl*, a transforming version of the gene carried in a retrovirus. The *c-abl* gene codes for a tyrosine kinase activity; this activity is essential for transforming potential in oncogenic variants. Deletion (or replacement) of the N-terminal region activates the kinase activity and transforming capacity. So the N-terminus provides a domain that usually regulates kinase activity; its loss may cause inappropriate activation.

Loss of tumor suppressors causes tumor formation

The common theme in the role of oncogenes in tumorigenesis is that increased or altered activity of the gene product is oncogenic. Whether the oncogene is introduced by a virus or results from a mutation in the genome, it is dominant over its allelic proto-oncogene(s). A mutation that activates a single allele is tumorigenic. Tumorigenesis then results from gain of a function.

Certain tumors are caused by a different mechanism: loss of both alleles at a locus is tumorigenic. Propensity to form such tumors may be inherited through the germline; it also occurs as the result of somatic change in the individual. Tumorigenesis then results from loss of function. Such cases identify tumor suppressors: genes whose products are needed for normal cell function, and whose loss of function causes tumors. The two best characterized genes of this class code for the proteins RB and p53.

Retinoblastoma is a human childhood disease, involving a tumor on the retina. It occurs both as a heritable trait and sporadically (by somatic mutation). It is often associated with deletions of band q14 of human chromosome 13. The *RB* gene has been localized to this region by molecular cloning.

Figure 39.11 illustrates the situation. Retinoblastoma arises when both copies of the *RB* gene are inactivated. In the inherited form of the disease, one parental chromosome carries an alteration in this region, usually a deletion. A somatic event in retinal cells that causes loss of the other copy of the *RB* gene causes a tumor. In the sporadic form of the disease, the parental chromosomes are normal, and both *RB* alleles are lost by (individual) somatic events.

Almost half the cases of retinoblastoma show deletions at the *RB* locus. In other cases, transcripts of the locus are either absent or altered in length. The protein product is absent from retinoblastoma cells. The cause of the tumor is therefore loss of protein function, usually resulting from mutations that prevent gene expression (as opposed to point mutations that affect function of the protein product). Loss of *RB* is involved also in other forms of cancer, including osteosarcomas and small cell lung cancers.

What is the molecular function of RB? It interacts with a variety of other proteins, including several tumor antigens: SV40 T antigen, adenovirus E1A, human papilloma virus E7. One possibility is that part of the oncogenicity of these proteins is due to

REVIEW ARTICLE Medical Biology 62: 304–317, 1984

AMPLIFICATION OF CELLULAR ONCOGENES IN CANCER CELLS

K. ALITALO

FROM THE DEPARTMENT OF VIROLOGY, UNIVERSITY OF HELSINKI, HELSINKI, FINLAND

ABSTRACT

Regulatory or structural alterations of cellular oncogenes have been implicated in the causation of various cancers. Oncogene alteration by point mutations can result in a protein product with strongly enhanced oncogenic potential. Aberrant expression of cellular oncogenes may be due to tumour-specific chromosomal translocations that dysregulate the normal functions of a proto-oncogene. Amplification of cellular oncogenes can also augment their expression by increasing the amount of DNA template available for the production of mRNA. It appears that amplification of certain oncogenes is a common correlate of the progression of some tumours and also occurs as a rare sporadic event affecting various oncogenes in different types of cancer. Amplified copies of oncogenes may or may not be associated with chromosomal abnormalities signifying DNA amplification: double minute chromosomes and homogeneously staining chromosomal regions. Amplified oncogenes, whether sporadic or tumour type-specific, are expressed at elevated levels, in some cases in cells where their diploid forms are normally silent. Increased dosage of an amplified oncogene may contribute to the multistep progression of at least some cancers.

KEY WORDS: CELLULAR ONCOGENES, GENE AMPLIFICATION, MULTISTEP CARCINOGENESIS, CLONAL SELECTION, KARYOTYPIC ABNORMALITIES, DOUBLE MINUTE CHROMOSOMES, HOMOGENEOUSLY STAINING CHROMOSOMAL REGIONS

DNA SEQUENCE AMPLIFICATION AND CYTOGENETIC ABNORMALITIES IN TUMOURS

Since its discovery in drug-resistant eukaryotic cells, somatic amplification of specific genes has been implicated in an increasing variety of adaptive responses of cells to environmental stresses (70, 79). Cytogenetic abnormalities, double minute chromosomes (dmin:s) associated with DNA amplification had already been discovered in tumour cells before the discovery of dmin:s and homogeneously staining chromosomal regions (HSR:s) in cells selected for drug-resistance (12, 24, 49, 50, 56). In metaphase spreads, dmin:s appear as small, spherical, usually paired chromosome — like structures that lack a centromere (Fig. 2). HSR:s stain with intermediate intensity throughout their length rather than with the normal pattern of alternating dark and light bands in both trypsin-Giemsa (Fig. 3A) and quinacrine dihydrochloride-stained preparations. Both kinds of abnormalities are occasionally found in metaphases of freshly isolated cancer cells but not of normal cells (8).

Dmin:s and HSR:s are apparently rare in tumour cells in vivo, although exact data are difficult to obtain since the abnormalities are easily missed in routine cytogenetic analysis (8, 42). Dmin:s and HSR:s have been described in most types of in vitro-cultured malignant tumour cells, with a notable frequency in neuroblastoma cell lines (11). Initial growth in cell culture apparently selects for tumour cells that contain either dmin:s or HSR:s. Moreover, in culture dmin:s are frequently lost, concomitant with the appearance of clonal populations of cells that have developed an HSR, suggesting that the two cytogenetic abnormaltities are alternative forms of gene amplification and that HSR:s may confer a selective advantage on cells over dmin:s (11, 70). It has been assumed that HSR:s can break down to firm dmin:s and that dmin:s can integrate into chromosomes to generate HSR:s (11, 23). Amplified genes may also occupy abnormally banding regions, ABR:s (51, 59). Experimental work on drug-resistant cells has shown that in the absence of a selection pressure (drug), dmin:s and the amplified genes that they contain are lost, whereas amplified DNA in the form of HSR:s is retained in the cells (71). This is explained by the fact that dmin:s are segregated unevenly in mitosis and frequently get lost from the nucleus due to

TABLE 1

Currently known oncogenes.

Retrovirus (example)	Oncogene	Gene product		
		Cellular location	Function of protein	Class
ONCOGENES FOUND IN RETROVIRUSES				
RSV	*src*	Plasma membrane	Tyrosine-specific protein kinases (*fgr* contains sequences homologous to actin)	Class Ia (Cytoplasmic tyrosine protein kinases)
Y73V	*yes*	Plasma membrane		
GR-FeSV	*fgr*	Plasma membrane		
Ab-MuLV	*abl*	Plasma membrane		
FuSV	*fps/fes*	Cytoplasm (plasma membrane?)		
ST-and GA-FeSV	*fes/fps*	Cytoplasm (cytoskeleton?)		
UR2V	*ros*			
AEV	*erb*-B	Plasma membrane and cytoplasmic membranes	EGF receptor's cytoplasmic domain	Class 1b (Class 1a-related proteins)
SM-FeSV	*fms*	Plasma membrane and cytoplasmic membranes	Cytoplasmic domain of a growth factor receptor?	
MH-2V	*mil/raf*	Cytoplasm	?	
3911-MSV	*raf/mil*	Cytoplasm	?	
Mo-MSV	*mos*	Cytoplasm	?	
SSV	*sis*	Secreted	PDGF-like growth factor	Class 2 (Growth factors)
Ha-MSV	Ha-*ras*	Plasma membrane	GTP-binding proteins	Class 3 (Cytoplasmic GTP:ases)
Ki-MSV	Ki-*ras*	Plasma membrane		
FBJ-MuSV	*fos*	Nucleus	?	Class 4 (Nuclear phosphoproteins)
OK-10V	*myc*	Nucleus	Nuclear matrix protein	
AMV	*myb*	Nucleus	?	
SKV 770	*ski*	Nucleus?	?	Unclassified
REV	*rel*	?	?	
AEV	*erb*-A	?	?	
E26V	*ets*	?	?	
ONCOGENES FOUND IN TUMOUR CELLS BUT NOT IN RETROVIRUSES				
Tumour cell				
Neuroblastoma	N-*ras*	Plasma membrane	GTP—binding	Class 3
Neuroblastoma	N-*myc*	?	?	Class 4
Small-cell lung cancer	L-*myc*	?	?	Class 4
Neuro-/Glioblastomas	*neu*	Plasma membrane	Growth factor receptor	Class 1b

their lack of centromeres, (49). HSR chromosomes carry centromeres and are therefore divided equally at mitosis. If dmin:s and HSR:s contain amplified genes that encode growth-stimulating protein products, it would follow that the more stable chromosomal form, the HSR, confers a greater selective growth advantage for cells. Although dmin:s and HSR:s have been described predominantly in tumour cells selected for resistance to cytotoxic drugs, it is also clear that dmin:s and HSR:s may be present in cancer cells before any form of therapy (8). It was in this setting that we and others first chose to explore the possible amplification of cellular oncogenes. (By definition, cellular oncogenes are normally innocent genetic loci which can be activated to transforming genes in various ways).[1]

DMIN:S AND HSR:S CONTAIN AMPLIFIED ONCOGENES

Table 2 summarizes the somatic amplifications of cellular oncogenes so far reported in

[1] It is not the purpose of this review to deal with all forms of DNA damage that have been found to activate cellular oncogenes. For the purpose of integrating the review into a coherent picture, however, the reader is given a list of known cellular oncogenes in Table 1 and the schematic Figure 1 illustrating the various ways in which the oncogenic potential of different proto-oncogenes can be activated.

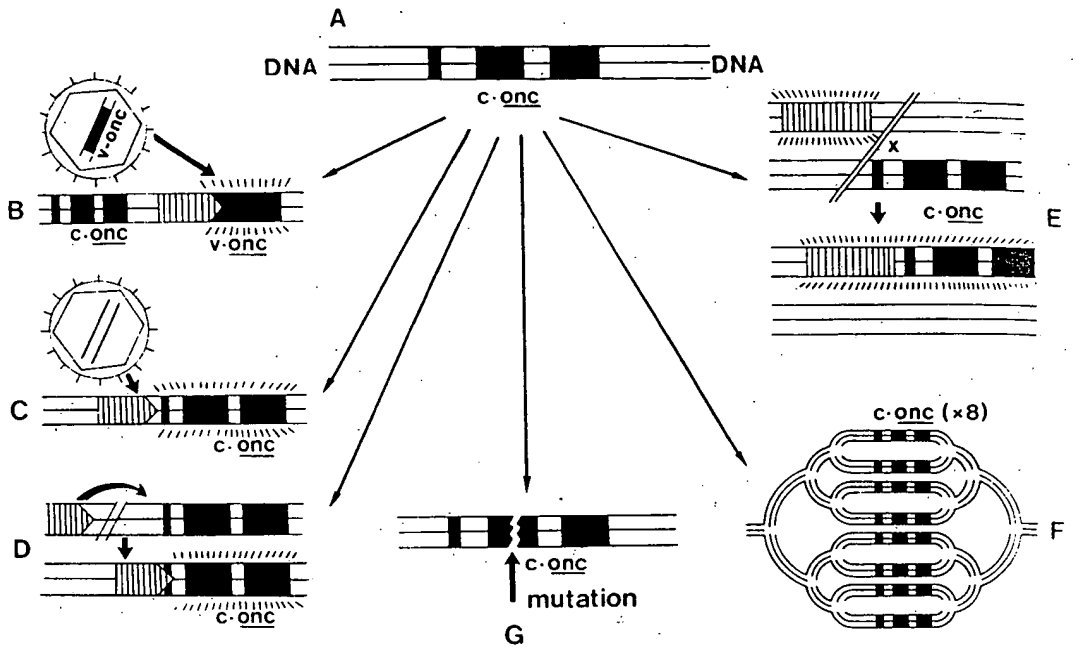


Fig. 1. Activation of cellular oncogenes. The haploid complement of a proto-oncogene is schematically depicted in A, composed of three exons (black boxes) in a segment of DNA. The different activated forms are schematically outlined in B—G. The abbreviation c-*onc* stands for cellular oncogene, v-*onc* viral oncogene, DNA sequences with associated strong promoter/enhancer functions are striated, and an actively transcribed gene is marked with radiations. B. Acute transforming retroviruses have the capacity to transduce cellular oncogenes (c-*onc*) into their genome, modify them and reinsert their activated oncogenes (v-*onc*) into the genome of host animal cells as a part of their proviral forms. The activity of the v-*onc* gene is greatly enhanced due to the associated promoter of the proviral long terminal repeat. Both increased dosage of the oncogene and structural mutations within its sequence may contribute to tumorigenesis. C. Slow transforming retroviruses without oncogenes replicate and reinsert their proviral copies into the host cell DNA during a latency period from infection to tumorigenesis. Tumor initiation through hyperplastic growth may begin, when the provirus integrates sufficiently close to a proto-oncogene to activate it through promoter or enchancer functions. It should be noted, however, that mutations have also been found in the oncogenes thus activated and that mutational damage to other oncogenes has been described in the resulting tumors. D. In some mouse plasmacytomas, a retrovirus-like DNA element (directing the synthesis of the so-called intracisternal A-type particles, IAPs) has been found in association with a transcriptionally activated oncogene c-*mos*. The IAP insertion also disrupts the 5' part of c-*mos* (64). E. In humans, as well as in animals, chromosome translocations may place proto-oncogenes into transcriptionally active regions of chromatin, where they may be activated. The details of this mechanism have not been worked out, but it is believed to occur for c-*myc* and c-*abl* genes in Burkitt lymphomas and Philadelphia-chromosome positive leukemias, respectively (35, 40). F. Increased amounts of oncogene-specific RNA and protein can also result from an excess of DNA template for transcription acquired through oncogene amplification. The present review concentrates primarily on this mechanism. G. Mutationally activated oncogenes have been found in nearly one fifth of human malignant tumours. Oncogene loci activated by somatic structural mutations are revealed by transfection experiments, where they are introduced into genetic background of nontumourigenic cultured immortalized cells. Several such transforming loci have been cloned and many of them belong to the c-*ras* oncogene family. It should be pointed out that both structural mutations and either increased expression or activation of a complementing oncogene may be required to achieve a fully tumorigenic phenotype (44).

tumour cells. Although the sampling of tumours is at present small, the finding of known cellular oncogenes among amplified DNA represented by dmin:s and HSR:s of cancer cells is provocative. Amplification has been found to affect at least five out of twenty known cellular oncogenes and the degree of gene amplification varies from five to many hundred-fold over the single haploid copies found in normal cells (see also ref. 18). The first amplification reported involved the c-*myc* oncogene (see Table 1) in a promyelocytic leukaemia cell line HL-60 (20, 25). The degree of c-*myc* amplification is between 8—32 fold both in the HL-60 cell line and in primary leukaemic cells from the patient (20, 25). Original clonal lines of HL-60 were later found to contain some dmin:s in culture but their number was insufficient to establish any clear correlation with amplified c-*myc*. Such a correlation, however, was discovered for c-*myc* amplification in a neuroendocrine cell line from

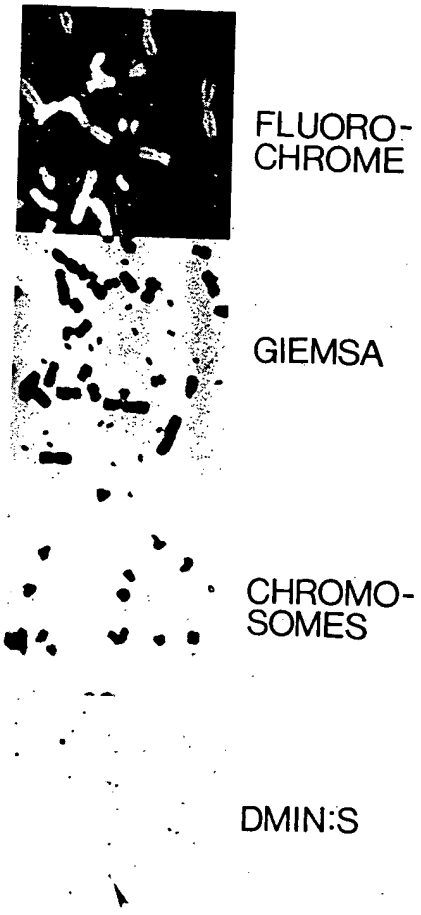


Fig. 2. Double minute chromosomes (arrowheads) in COLO 320DM colon carcinoma cells. A. The dmin:s are resolved as paired dots among normal chromosomes in this fluorescent, benzimidazole-stained preparation B—D. Purification of dmin:s by differential centrifugations. B. The starting material, C. Chromosome fraction, D. Purified dmin:s (Donna George and the author, unpublished data and ref. 52).

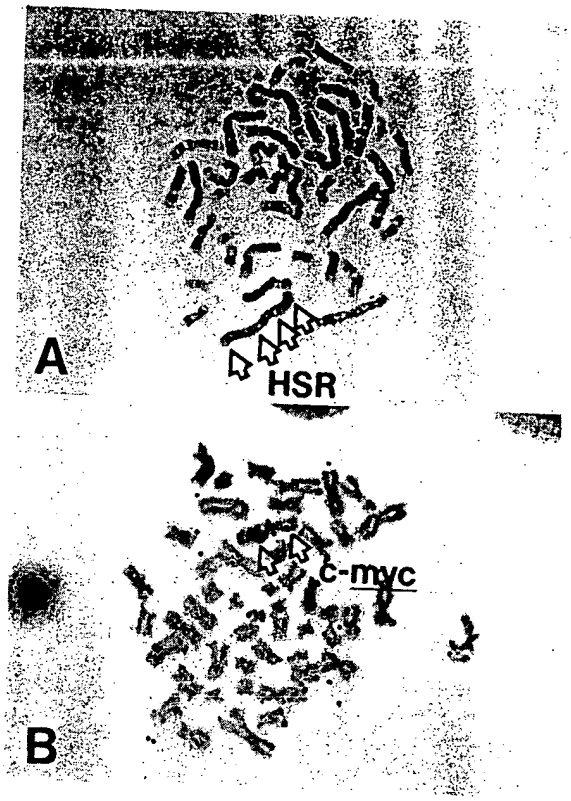


Fig. 3. A. The homogeneously staining regions (HSR) in the G-banded HSR-marker chromosome comprise a major portion of both its long and short arms. The HSR-marker chromosome has evolved from an X-chromosome (52 and unpublished data of C. C. Lin and the author). B. The about 30-fold amplified copies of the c-*myc* oncogene in COLO 320 cells were found to be located to dmin:s and HSR:s. The latter is shown here by in situ-hybridization (5, 52).

a colon carcinoma, COLO 320 (5). In these cells, the approximately 30-fold amplified c-*myc* copies were mapped either to HSR:s of a marker chromosome (5, Fig 3B) or to dmin:s (52), depending on the particular subline studied. Since dmin:s were already present in the primary tumour cells from this colon carcinoma (63), it is likely that c-*myc* had also been amplified during growth of the tumour in vivo. Similarly, amplified copies of the c-Ki-*ras* oncogene were mapped to dmin:s and HSR:s of a mouse adrenocortical tumor Y1 (74). An extensive search for changes in other oncogenes and tumour cells has since revealed amplifications that do not show up as dmin:s or HSR:s. Thus, for example, the c-*myb* oncogene is amplified in a characteristic marker chromosome of a colon carcinoma without evidence of HSR:s (ref. 6, Fig. 4) and in other tumours, the amplified c-*abl* and c-*myc* oncogene loci map to abnormally banding regions (ABR:s) in translocated or resident chromosomal segments, respectively (59, 76).

TRANSLOCATIONS AND REARRANGEMENTS MAY ACCOMPANY ONCOGENE AMPLIFICATION

The evolution and progression of the karyotype of tumour cells is complicated (see ref. 68). Concomitant with amplification, DNA sequences acquire an increased mobility in the genome with extrachromosomal intermediates

TABLE 2

*Sporadic and tumour-specific amplification of cellular oncogenes.**

Tumour cells	Oncogene	Fold amplified	Chromosomal location of amplified gene	Expression elevated	Remarks	References
Sporadic:						
HL60 (acute promyelocytic leukaemia, M3)	c-*myc*	20×	8q(ABR)	Yes	Amplification present in primary leukaemic cells	20, 25, 59
COLO320 (colon carcinoma)	c-*myc*	30×	dmin, HSR	Yes	Part of the amplified c-*myc* sequences rearranged	4, 5, 52
Y1 (adrenocortical tumour)	c-Ki-*ras*	50×	dmin, HSR	Yes	Levels of p 21$^{c\text{-}Ki\text{-}ras}$ protein elevated	74
COLO201/205 (colon carcinoma)	c-*myb*	10×	mar1	Yes	Patient treated with 5-fluorouracil prior to culturing of the tumour cells	4, 6,88
K562 (chronic myelogenous leukaemia, CML)	c-*abl*	10×	mar(ABR)	Yes	C_λ coamplified in the marker that may be derived from chromosome 22, c-*abl* protein-associated tyrosine kinase activated	21, 22, 41, 54, 76
A431 (epidermoid carcinoma)	c-*erb*B	15—20×	n.d.	Yes	Amplification linked to chromosome 7 translocation and sequence rearrangements Amount of protein product, the EGF receptor, elevated	82 (see 36)
ML1—3 (acute myeloid leukaemia, M2)	c-*myb*	5—10×	n.d.	Yes	Abnormalities of chromosome 6q22—24, where c-*myb* is normally located	34, 61, 91
SK BR-3 (breast carcinoma)	c-*myc*	10×	n.d.	Yes		43
SEWA (polyoma virus-induced mouse tumour)	c-*myc*	30×	n.d.	Yes	Cells have dmin:s depending on culture conditions; c-*myc* amplification correlates with growth as a tumour	Manfred Schwab, personal communication
Lu-65 (lung giant cell carcinoma)	c-*myc* c-Ki-*ras*	8× 10×	n.d. n.d.	n.d. n.d.	At least some copies of c-Ki-*ras* mutated	80
Primary leukemic cells from an acute myeloid leukemia (M2) patient	c-*myc*	33×	n.d.	n.d.		Unpublished data of the author and A. de la Chapelle
Tumour-specific:						
small-cell lung cancer	c-*myc* L-*myc*, N-*myc*	up to 80×	n.d.	Yes	Most amplifications in the variant phenotype of SCLC	53, 69
Neuroblastomas	*N-myc*	up to 250×	dmin, HSR	Yes	N-*myc* also amplified in primary tumours of advanced grade	14, 48, 72, 73, 75
Glioblastomas	c-*erb*B	—	—	—	Rearrangements also found personal communication	Josef Schlessinger, personal communication

n.d. = not determined, mar = marker chromosome, M2, M3 refer to the French-American-Brittish classification of acute myeloid leukemias.

* At least one case of oncogene amplification in normal germ-line cells has been found (18).

visualized as dmin:s, transpositions and translocations to other chromosomal segments, etc. (see 70 for references). There may not be preferred chromosomal sites for the apparent reintegration of dmin:s as HSR:s (75). In at least one case, however, an oncogene may have been caught amplifying in situ in its resident chromosomal site (59). The finding of moderately amplified oncogenes also in chromosomal sites lacking HSR:s suggests that

Fig. 4. Localization of amplified c-*myb* in COLO 201/205 cells by in situ hybridization. Shown is a characteristic, large marker chromosome (mar1) with G-banding (A) and associated c-*myb* autoradiographic grains (B). Note the absence of HSR:s. Mar1 has probably evolved from chromosome number 6, the resident site of the c-*myb* oncogene in normal cells (34, 88, 91). (Robert Winqvist and the author, unpublished data).

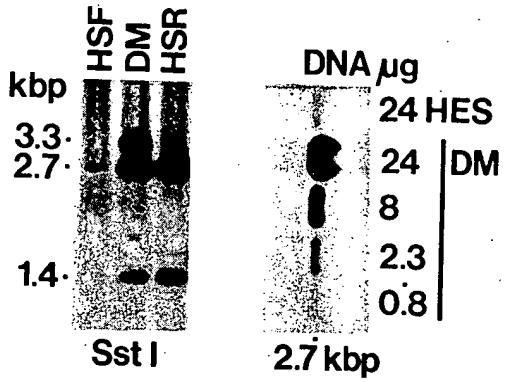


Fig. 5. Amplification and rearrangement of c-*myc* in COLO 320 cells. 10 µg of cellular DNA was digested with Sst I, electrophoresed, blotted and probed with a v-*myc* Pst I fragment (ref. 2, left panel). Fragments of 2.7 kbp and 1.4 kbp are seen in both normal and amplified c-*myc* DNA. The 3.3 kbp fragment is derived from a DNA segment of unknown origin translocated to the 5′ region of c-*myc* with a concomitant deletion of its first exon (unpublished data of Manfred Schwab and the author). HSF, human skin fibroblasts; DM, COLO 320 DM cells; HSR, COLO 320 HSR cells. Different amounts of DNA from COLO 320 DM cells as indicated were mixed with calf thymus DNA to give 24 µg of total DNA, cleaved with Sst I, electrophoresed, blotted and probed with a fragment of 3′human c-*myc* sequences. The intensities of the 2.7 kbp c-*myc* fragment in different samples were compared to assess its copy number, estimated to be about 30 (5).

(onco)gene amplification may be more common than the structural alterations shown by chromosome banding and microscopy (6, 88).

In at least three cases reported amplification has been accompanied by a DNA rearrangement of the oncogene (5, 20, 82). In the colon carcinoma COLO 320 both damaged and normal versions of the c-*myc* gene are amplified (5). Although individual cell clones have not yet been examined, our unpublished experiments suggest that the same dmin-containing cells harbor and express both normal and rearranged forms of c-*myc*. The normal version of the amplified gene, however, predominates in COLO 320 cells containing HSR:s; the rearranged version is present only in what appears to be a single copy (Fig. 5). In the chronic myeloid leukaemia (erythroleukaemia) cell line K562 an amplified DNA segment consists of portions of both the c-*abl* oncogene and the immunoglobulin C_λ locus (76). In both cases abnormal transcripts are produced from the rearranged amplified oncogenes (Fig. 6 and ref. 22). In K562 cells, the abnormal c-*abl* oncogene product has also been activated as a tyrosine protein kinase (41). It is not known, however, whether structural alterations of the genes preceded amplification or whether they were acquired during the process of gene amplification. It seems likely that a chromosomal translocation of c-*abl* to the C_λ locus preceded DNA amplification in the K562 cells, since all amplified copies were also rearranged (21), with the change reminiscent of the Philadelphia translocation (t(9, 22)) found in most chronic myeloid leukaemia tumours (35, 66—68). Although they have not been sequenced, other reported cases of amplified oncogenes are apparently normal on basis of mapping with restriction endonucleases (see Table 2). Therefore we cannot at present view mutation as a necessary companion of oncogene amplification.

THE MECHANISMS OF GENE AMPLIFICATION

The mechanisms of gene amplification and the structure of the amplified DNA have been worked out mainly in experimental settings involving selection for drug-resistance in cell culture (70). Although the mechanisms are still incompletely known and may vary in different cases, some general features have emerged.

A spontaneous degree of illegitimate DNA replication seems to exist in normal cells so that various segments of DNA are replicated more than once during a single cell cycle (37). In unselective conditions this DNA is probably lost e.g., through formation of micronucleae

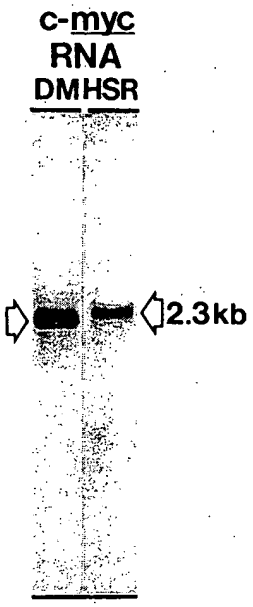


Fig. 6. Comparison of the electrophoretic mobilities of c-*myc* mRNA:s from COLO 320 DM and HSR cells. The size of the normal c-*myc* mRNA is 2.3 kb. The rearranged c-*myc* locus in DM cells (see Fig. 5) seems to be predominantly expressed giving rise to a shortened RNA.

because the newly synthesised extra copies of DNA are not covalently linked to chromosomal DNA of mitotic cells (65, 71). If, however, there is a selection pressure to retain an increased gene dosage, progressive multiplication of gene copy number results. The incidence of cells bearing amplified genes under conditions of cytotoxic selection can vary by two orders of magnitude and is greatly increased by the presence of mitogenic substances (hormones or tumour promoters) during selection (10, 84, 85) or certain carcinogenic or cytotoxic agents before selection (15, 55, 79, 80, 81, 85). An interesting hypothesis suggested by Varshavsky (84, 85) supposes that the origins of DNA replication "fire" (initiate replication) illegitimately several times during a single cell cycle and that this kind of "replicon misfiring" may be increased by substances such as tumour promoters and mitogenic hormones (10, 84, 85). Mariani and Schimke (55) point out that most of the cytotoxic agents that increase the incidence of gene amplification are inhibitors of DNA synthesis. Aberrant replication is known to take place after transient inhibition of DNA synthesis and this response can lead to gene amplification (46, 47, 55, 90). Mitogenic hormones probably increase disproportionate DNA replication, but they also enhance the colony forming efficiency of drug-resistant cells in selective conditions (10).

According to the studies of Axel and his collaborators (65), the multiple cycles of unscheduled DNA replication at a single locus during a single cell cycle result in a structure schematically outlined in Fig. 1F. The hydrogen-bonded amplified copies of DNA depicted in Fig. 1F must resolve into a tandem linear array before the next mitosis. This may well occur by homologous recombination between any one of several repeated sequences within the amplified domain (45, 65). Part of the recombinations would lead to extrachromosomal circles possessing an origin for replication (16, 62); these could be the precursors of dmin:s. The unequal recombinations mean that the resolved linear structure consists of tandemly repeated but heterogeneous units. According to Axel's model a gradient of amplification is formed so that centrally located sequences are amplified more than sequences distal to the origin of replication (65). This also has, in fact, been found to explain the large, complex DNA domain amplified in neuroblastoma cells in vivo (38, see also below).

The chromosomal site of integration of transfected genes significantly affects the frequency and cytogenetic result of their experimentally induced amplification (83). The amplification frequency in some transformants has been found to be 100-fold that of the others (83). This suggests that there also are preferred chromosomal positions for amplification of host cellular genes and that chromosomal rearrangements may facilitate gene amplification by positioning chromosomal sequences in a favorable array. In respect of the structural properties of the sequences involved in gene amplification, recombinatorially active regions have been implicated in experimental cases. DNA rearrangements involving restriction fragment length polymorphisms and variation in gene copy number have been detected in the human genome between clusters of short repetitive interspersed DNA sequences called Alu family DNA-sequences (17). Such inter-Alu sequences have also been detected in an extrachromosomal DNA form, including covalently closed circles (17, 78). The copy number of inter-Alu sequences apparently varies in an age- and tissue-specific manner (17, 78), but any comprehensive analysis of the phenomenon in human tumours is not yet available. It is also not yet clear whether these kinds of repetitive sequences are involved in generating amplified oncogene sequences in dmin:s or HSR:s in tumours.

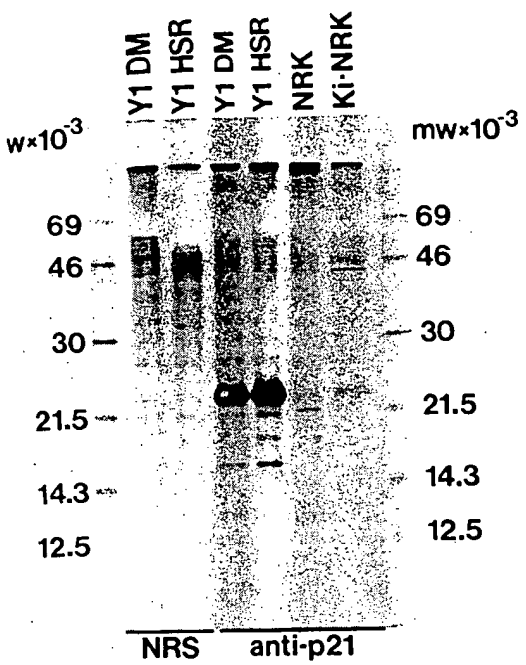


Fig. 7. Elevated levels of the p21$^{c\text{-}Ki\text{-}ras}$ protein in Y1 cells (74). The Y1 DM and HSR cells which harbor a 50-fold amplified c-Ki-*ras* oncogene (74) and control cells were labeled with [^{35}S]-methionine and the p21$^{c\text{-}Ki\text{-}ras}$ protein was immunoprecipitated, as detailed (74), with normal rat serum (NRS) or rat monoclonal anti-p21 serum. The proteins were electrophoresed in a 15 % polyacrylamide gel in the presence of SDS. In addition to a major p21 band, a labeled band at about 16 kd was present in the immunoprecipitates. The amount of radioactivity in p21 was about 50 fold that in normal rat kidney cells. The Kristen sarcoma virus-transformed rat kidney cells (obtained from the American Tissue Culture Collection) also yielded unexpectedly low amounts of the v-Ki-*ras* protein.

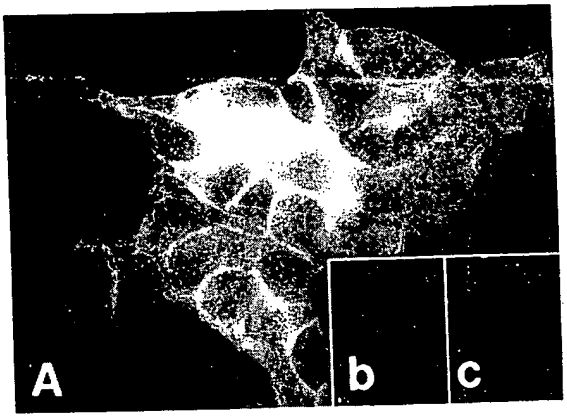


Fig. 8. A. Indirect immunofluorescence for p21$^{c\text{-}Ki\text{-}ras}$ in Y1 DM cells. Similar fluorescence of the plasma membrane was obtained for the Y1 HSR cells. Inset (b) shows control staining with normal rat serum and inset (c) staining of normal rat kidney cells with the monoclonal antibody against p21.

CARCINOGEN-INDUCED GENE AMPLIFICATION AND CLONAL SELECTION OF CANCER CELLS

Although cell sorting experiments have shown a basal spontaneous rate of gene amplification in eukaryotic cells (37), this can be increased severalfold by metabolic inhibitors or cytotoxic agents (15, 37, 70, 81, 85). In many respects the latter response is reminiscent of the so-called SOS-response elicited in bacteriae by noxious stimuli (see 28). In a teleological context, the rapid induction of gene amplification that apparently occurs frequently through extrachromosomal intermediates may provide cells with genetic material for subsequent selective pressures operating in harmful conditions (60). In cancer cells, the mechanism may enhance the emergence of clonal populations of cells with increasingly malignant properties (58, 60). Such genetic instability of cancer cells is clearly enhanced, leading to the rapid evolution of increasingly malignant tumour cell populations (19, 58). A serious question of practical importance is whether drug resistance in treated patients also selects cells that have an enhanced ability to amplify (onco)genes important for growth and progression of the tumour (84, 85). It is also possible that some of the carcinogenic insults caused by mutagens are only expressed as a result of subsequent amplification events induced by tumour promoters (84, 85) or facilitated by hormones in, say replicating epithelial cells (10). The persistence of dmin:s in some tumours suggests that there is a selection pressure for their retention (8, 9, 11, 23). Amplified DNA in dmin:s must contain an origin for DNA replication (62) and must be selected for in daughter cell populations, where it is unevenly segregated (71). In the absence of such a selection pressure dmin:s are lost (71). In at least one study the length of an HSR has been found to increase during a selection of malignant cells for enhanced tumourigenicity (30).

The amplified c-*erb*B gene in A431 cells codes for epidermal growth factor receptor (27). The abundant amounts of receptor protein on A431 cell surface may, however, provide the cells with an abnormal growth response (31). A naive supposition is that the amplified sequences in dmin:s and possibly in HSR:s of tumours contain growth-promoting genes (see 36 for references). This seems to fit well with

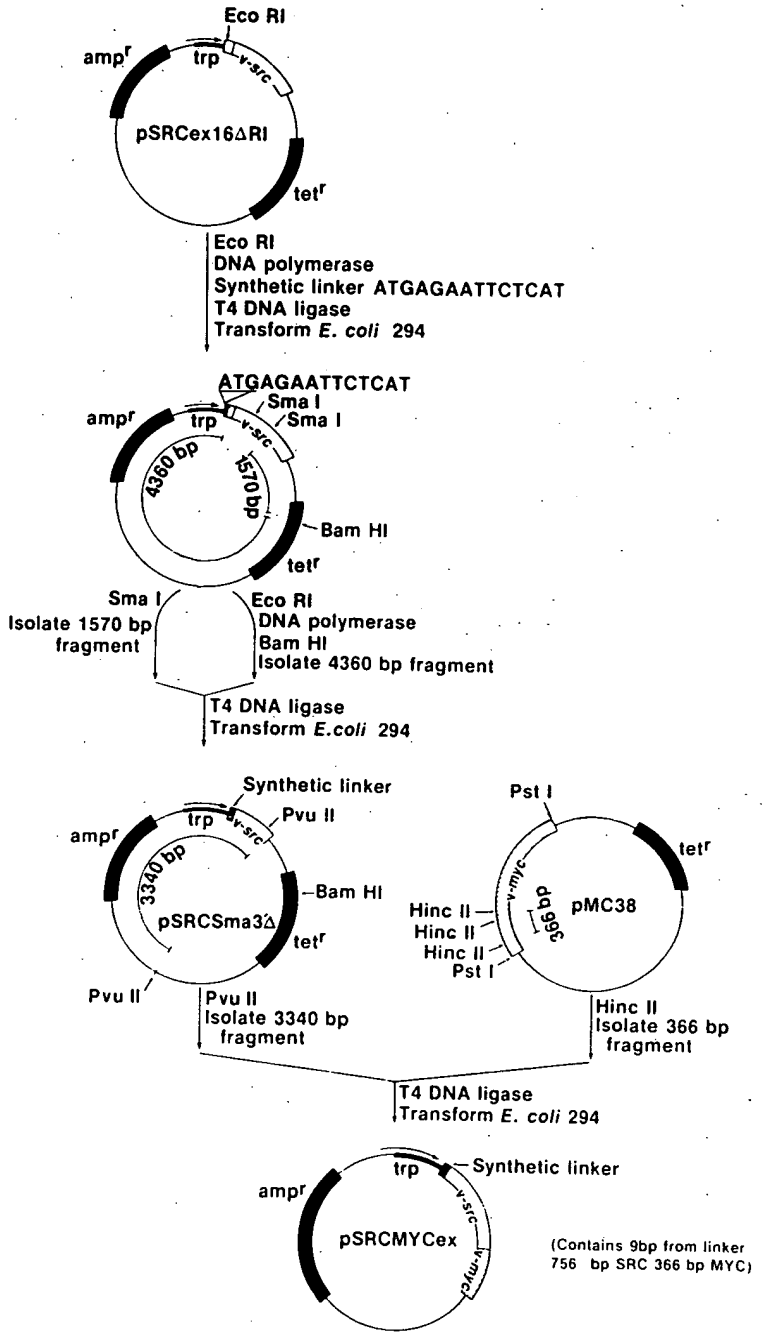


Fig. 9. Construction of a v-*myc* expression vector. A synthetic linker (ATGAGAATTCATCAT) containing a translational initiation codon was inserted downstream from the *trp* promoter in the pSRC ex16 RI expression vector described previously (see ref. 3). Approximately one-half of the v-*src* sequences coding for the aminoterminal portion of pp60$^{v\text{-}src}$ protein were then deleted and the remaining portion ligated in translational codon frame with the synthetic ATG. A Hinc II fragment of v-*myc* from plasmid clone MC 38 (nucleotides 320—685 in the v-*myc* sequence in ref. 2) was ligated downstream form remaining v-*src* sequences in continuity with its reading frame. The resulting product contained 3 amino acids from the synthetic linker, 252 amino acids encoded by the 756 base pair fragment from Sma I to Pvu II restriction sites in v-*src* DNA, 122 amino acids from the v-*myc* and 6 amino acids (corresponding to nucleotides 2968—2085) from the pBR322 vector (3).

recent findings on amplified oncogenes, though in many cases the search for an amplified oncogene is still continuing. Even positive findings do not mandate a role for amplified cellular oncogenes, however, because the domain of amplified DNA is inevitably much larger than a single genetic locus (e.g. 38).

ENHANCED EXPRESSION OF AMPLIFIED ONCOGENES

In all cases where they have been studied, the amplified oncogenes have been found abundantly expressed at the RNA level, roughly in proportion to the amount of DNA amplification (see Table 1). Described cases of elevated RNA expression include examples of abnormal (5, 22) and ectopic (6) transcription. In at least four cases this enhancement is not limited to synthesis of RNA (31, 33, 41, 74, 82). The Y1 cells that have amplified c-Ki-*ras* contain exceptionally large amounts of its protein product situated on the plasma membrane (ref. 74, Fig. 7 and 8). High amounts of the c-*myc* encoded protein are also found in COLO 320 cells that have amplified the gene (33). The *myc* oncogenes have recently been shown to encode nuclear proteins (ref. 1, 3, 26, 29, 32, 33, Fig. 9—11). Both the expression of the c-*myc* mRNA (39) and the subcellular localization of myc proteins are linked to the cell cycle (ref. 89, Fig. 12). It may be that elevated expression of specific c-*myc* functions is necessary for cell cycle progression and the growth transformation aspect of the phenotype of cancer cells that may contribute to tumour progression (7, 36). Elevated expression of c-*myc* has been shown to replace in part platelet-derived growth factor in induction of competence for DNA replication (7). Generally, enhanced expression of an oncogene could be a necessary prerequisite for acquisition of a growth advantage by cells having extra copies of the gene. This effect could also be the principal contribution of amplification to tumourigenesis.

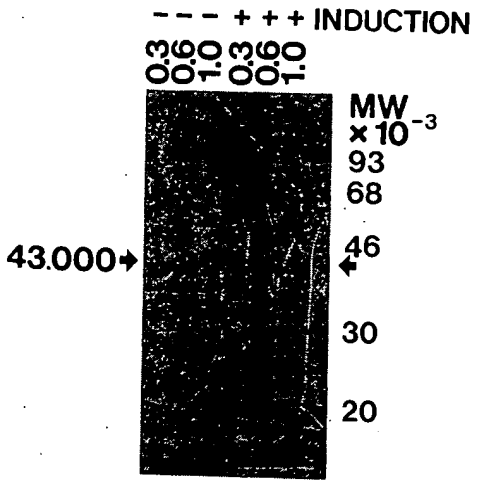


Fig. 10. *E. coli* 294 was transfected with the hybrid v-*src*, v-*myc* plasmid outlined in Fig. 9 and ampicillin-resistant bacterial colonies were checked for the production of a 43,000 m.w. bacterial v-*myc* protein after induction by growth to different optical densities in minimal essential medium (M9, induction+) or complete medium (LB, induction—) (3).

TUMOUR CELL AND STAGE SPECIFICITY OF ONCOGENE ACTIVATION AND AMPLIFICATION

Tumour cell specificity of oncogene amplification has been found in three malignancies. The c-*myc*, L-*myc* or N-*myc* oncogene is amplified in most cases of the variant form of small-cell lung cancer cells (53, 69), c-*erb*B is amplified in several glioblastomas (Josef Schlessinger, personal communication) and the putative N-*myc* oncogene is amplified in about half of grade III and IV neuroblastomas (14, 72, 73, 75). In addition to HSR:s, small-cell lung cancers and neuroblastomas frequently show a

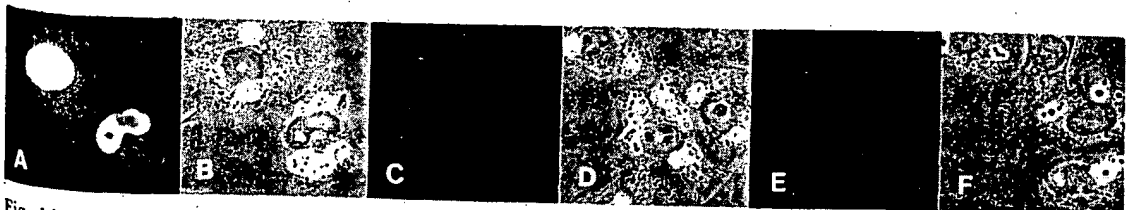


Fig. 11. Indirect immunofluorescence for the v-*myc* protein and phase contrast microscopy of myelocytomatosis virus-transformed quail cells (3). A. Quail cells transformed with the MC-29 virus (Q8 cells). Anti-*myc* protein staining. B. Phase contrast microscopy of field in A. C. Q8 cells stained with anti-*myc* protein antiserum that has been blocked with the immunogen. D. Phase contrast microscopy of field in B. E. Q8 cell stained with preimmune rabbit serum. F. Phase contrast microscopy of field in E.

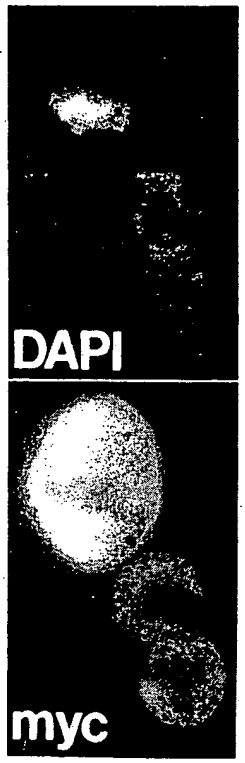


Fig. 12. Fluorescent staining for DNA and *myc* protein in myelocytomatosis virus-transformed quail cells. In interphase cells, the *myc* protein is confined to the nucleae. In the mitotic cell, *myc* fluorescence is distributed throughout the cell unlike fluorescence for chromatin, which is compacted to chromosomes in the metaphase plate. In fact, there is less *myc* fluorescence associated with chromatin than with the rest of the cell. DAPI, diaminophenylindole DNA stain. The anti-*myc* protein rabbit antiserum was used in a 1/200 dilution (ref. 89).

deletion of a portion of the short arm of chromosome 1 (13) and chromosome 3 (86, 87), respectively, in karyological examination. Two kinds of changes have also been described in different neuroblastoma oncogenes. The first is a mutation in the N-*ras* gene, an activated oncogene that was discovered because of its relation to other *ras* genes and transforming activity in transfection experiments (77). The second is amplification of a distant homologue of the c-*myc* gene called N-*myc* (72, 73, 75). Although the transforming potential of the N-*myc* gene has not yet been established, its consistent presence in a core segment of amplified neuroblastoma DNA (38, 57, 72, 73, 75) and its elevated expression in most retinoblastomas (48) suggests its oncogenic nature.

Taya et al. (80) have recently described a human lung giant cell carcinoma grown in nude mice, where both c-Ki-*ras* and c-*myc* oncogenes were amplified about 10-fold. Besides, sequencing studies indicated that at least some of the amplified c-Ki-*ras* copies were also mutationally activated in the 12th codon. These results fit to the multistage theory of cancer development and progression (see 58). Apparently co-operating lesions in cellular oncogenes accumulate during tumour growth and selection and increase the malignant potential of the tumour cells (44).

When does oncogene amplification come into play during tumourigenesis? Gene amplification may not be any initiating event in carcinogenesis. Amplification and enhanced expression of c-*myc* and N-*myc* may occur during the progression of human carcinoma of the lung and neuroblastoma cells to a more malignant phenotype (14, 53, 73). There may be, however, no mandatory sequence of oncogene amplifications for the genesis of any particular tumor. Amplification of an oncogene could play its part in malignant progression of already initiated cells whenever it happened to occur.

ACKNOWLEDGEMENTS

I thank my colleagues Manfred Schwab, Gerard Evan, J. Michael Bishop, Robert Winqvist, Kalle Saksela, Jorma Keski-Oja, C. C. Lin, Arthur Levinson, Wendy Colby and Donna George for collaboration, Ron Ellis for monoclonal antibodies against p21 proteins and Stephen Hann and Robert Eisenman for communicating their results before publication. The studies in the author's laboratory were supported by the Finnish Cancer Research Fund and by the Academy of Finland.

REFERENCES

1. *Abrams HD, Rohrschneider LR, Eisenman RN:* Nuclear location of the putative transforming protein of avian myelocytomatosis virus. Cell 29: 427–439, 1982
2. *Alitalo K, Bishop JM, Smith DH, Chen EY, Colby WW, Lenvinson AD:* Nucleotide sequence of the v-*myc* oncogene of avian retrovirus MC29. Proc Natl Acad Sci USA 80: 100–104, 1983
3. *Alitalo K, Ramsay G, Bishop JM, Ohlsson-Pfeifer S, Colby WW, Levinson AD:* Identification of nuclear proteins encoded by viral and cellular *myc* oncogenes. Nature 306: 274–277, 1983
4. *Alitalo K, Saksela K, Winqvist R, Schwab M, Bishop JM:* Amplification and aberrant expression of cellular oncogenes in human colon cancer cells. In: Genes and cancer. Ed. J. M. Bishop and J. Rowley. Alan Liss Co, New York, in press
5. *Alitalo K, Schwab M, Lin CC, Varmus HE, Bishop JM:* Homogeneously staining chromosomal regions con-

tain amplified copies of an abundantly expressed cellular oncogene (c-*myc*) in malignant neuroendocrine cells from a human colon carcinoma. Proc Natl Acad Sci USA 80: 1707–1711, 1983
6. *Alitalo K, Winqvist R, Lin CC, de la Chapelle A, Schwab M, Bishop JM:* Aberrant expression of an amplified c-*myb* oncogene in two cell lines from a colon carcinoma. Proc Natl Acad Sci USA 81: 4534–4538, 1984
7. *Armelin HA, Armelin MCS, Kelly K, Stewart T, Leder P, Cochran BH, Stiles CD:* Functional role for c-*myc* in mitogenic response to platelet-derived growth factor. Nature 310: 655–660, 1984
8. *Barker PE:* Double Minutes in human tumor cells. Cancer Genet Cytogenet 5: 81–94, 1982
9. *Barker PE, Drwinga HL, Mittelman WN, Maddox AM:* Double minutes replicate once during S phase of the cell cycle. Exp Cell Res 130: 353–360, 1980
10. *Barsoum J, Varshavsky A:* Mitogenic hormones and tumor promoters greatly increase the incidence of colony-forming cells bearing amplified dihydrofolate reductase genes. Proc Natl Acad Sci USA 80: 5330–5334, 1983
11. *Biedler JL, Meyers MB, Spengler BA:* Homogeneously staining regions and double minute chromosomes, prevalent cytogenetic abnormalities of human neuroblastoma cells. Adv Cell Neurobiol 4: 268–301, 1983
12. *Biedler JL, Spengler BA:* Metaphase chromosome anomaly: association with drug resistance and cell-specific products. Science 191: 185–187, 1976
13. *Brodeur GM, Green AA, Hayes FA, Williams KJ, Williams DL, Tsiatis AA:* Cytogenetic features of human neuroblastomas and cell lines. Cancer Res 41: 4678–4686, 1981
14. *Brodeur GM, Seeger RC, Schwab M, Varmus HE, Bishop JM:* Amplification of N-*myc* in Untreated Human Neuroblastomas Correlates with Advanced Disease Stage. Science 224: 1121–1124, 1984
15. *Brown PC, Tlsty TD, Schimke RT:* Enhancement of methotrexate resistance and DHFR gene amplification by treatment of 3T6 cells with hydroxyurea. Mol Cell Biol 3: 1097–1107, 1983
16. *Bullock P, Botchan M:* Molecular events in the excicion of SV40 DNA from the chromosomes of cultured mammalian cells. In: Gene amplification. Ed. R. T. Schimke. Cold Spring Harbor Lab 215–230, 1982
17. *Calabretta B, Robberson DL, Barrera-Saldana HA, Lambrou TP, Saunders GF:* Genome instability in a region of human DNA enriched in Alu repeat sequences. Nature 296: 219–225, 1982
18. *Chattopadhyay SK, Chang EH, Lander MR, Ellis RW, Scolnick EM, Lowy DR:* Amplification and rearrangement of *onc* genes in mammalian species. Nature 296: 361–363, 1982
19. *Cifone MA, Fidler IJ:* Increasing metastatic potential is associated with increasing genetic instability of clones isolated from murine neoplasms. Proc Natl Acad Sci USA 78: 6949–6952, 1981
20. *Collins S, Groudine M:* Amplification of endogenous *myc*-related DNA sequences in a human myeloid leukemia cell line. Nature 298: 679–681, 1982
21. *Collins SJ, Groudine MT:* Rearrangement and amplification of c-*abl* sequences in the human chronic myelogenous leukemia cell line K-562. Proc Natl Acad Sci USA 80: 4813–4817, 1983
22. *Collins SJ, Kubonishi I, Miyoshi I, Groudine MT:* Altered transcription of the c-*abl* oncogene in K-562 and other chronic myelogenous leukemia cells. Science 225: 72–74, 1984
23. *Cowell JK:* Double minutes and homogeneously staining regions: Gene amplification in mammalian cells. Ann Rev Genet 16: 21–52, 1982
24. *Cox D, Yuncken C, Spriggs AI:* Numerous minute chromatin bodies in malignant tumours of childhood. Lancet I: 55–58, 1965
25. *Dalla-Favera RD, Wong-Staal F, Gallo RG: Onc* gene amplification in promyelocytic leukemia cell line HL-60 and primary leukaemic cells of the same patient. Nature 299: 61–63, 1982
26. *Donner P, Greiser-Wilke I, Moelling K:* Nuclear Localization and DNA binding of the transforming gene product of avian myelocytomatosis virus. Nature 296: 262–266, 1982
27. *Downward J, Yarden Y, Mayes E, Scrace G, Totty N, Stockwell P, Ullrich A, Schlessinger J, Waterfield MD:* Close similarity of epidermal growth factor receptor and v-*erb*B oncogene protein sequences. Nature 307: 521–527, 1984
28. *Echols H:* SOS-functions, cancer and inducible evolution. Cell 25: 1–2, 1981
29. *Eisenman RN, Tachibana CY, Abrams HD, Hann SR:* v-*myc* and c-*myc*-Encoded proteins are associated with the nuclear matrix. Submitted
30. *Gilbert F, Balaban G, Brangman D, Herrmann N, Lister A:* Homogeneously staining regions and tumorigenicity. Int J Cancer 31: 765–768, 1983
31. *Gill GN, Lazar CS:* Increased phosphotyrosine content and inhibition of proliferation in EGF-treated A431 cells. Nature 293: 305–307, 1981
32. *Hann SR, Abrams HD, Rohrschneider LR, Eisenman RN:* Proteins encoded by v-*myc* and c-*myc* oncogenes: Identification and localization in acute leukemia virus transformants and bursal lymphoma cell lines. Cell 34: 789–798, 1983
33. *Hann SR, Eisenman RN:* Two proteins encoded by the human c-*myc* oncogene: Differential expression in neoplastic cells. Mol Cell Biol 4: 2486–2497, 1984
34. *Harper ME, Franchini G, Love J, Simon MI, Gallo RC, Wong-Staal F:* Chromosomal sublocalization of human c-*myb* and c-*fes* cellular onc genes. Nature 304: 169–171, 1983
35. *Heisterkamp N, Stephenson JR, Groffen J, Hansen PF, de Klein A, Bartman CR, Grosveld G:* Localization of the c-*abl* oncogene adjacent to a translocation break point in chronic myelocytic leukaemia. Nature 306: 239–242, 1983
36. *Heldin C-H, Westermark B:* Growth factors: Mechanism of action and relation to oncogenes. Cell 37: 9–20, 1984
37. *Johnston RN, Beverley SM, Schimke RT:* Rapid spontaneous dihydrofolate reductase gene amplification shown by fluorescence-activated cell sorting. Proc Natl Acad Sci USA 80: 3711–3715, 1983
38. *Kanda N, Schreck R, Alt F, Bruns G, Baltimore D, Latt S:* Isolation of amplified DNA sequences from IMR-32 human neuroblastoma cells: Facilitation by fluoroscence-activated flow sorting of metaphase chromosomes. Proc Natl Acad Sci USA 80: 4069–4073, 1983
39. *Kelly K, Cochran BH, Stiles CD, Leder P:* Cell-specific regulation of the c-*myc* gene by lymphocyte mitogens and platelet-derived growth factor. Cell 35: 603–610, 1983
40. *Klein G:* Specific chromosome translocations and the genesis of B-cell derived tumors in mice and men. Cell 32: 311–315, 1983
41. *Konopka JB, Watanabe SM, Witte ON:* An alteration of the human c-*abl* protein in K562 leukemia cells unmasks associated tyrosine kinase activity. Cell 37: 1035–1042, 1984
42. *Kovacs G:* Homogeneously staining regions on marker

chromosomes in malignancy. Int J Cancer 23: 299—301, 1979
43. *Kozbor D, Croce CM:* Amplification of the c-*myc* oncogene in one of five human breast carcinoma cell lines. Cancer Res 44: 438—441, 1984
44. *Land H, Parada LF, Weinberg RA:* Tumorigenic conversion of primary embryo fibroblasts requires at least two cooperating oncogenes. Science 222: 771—778, 1983
45. *Latt SA:* Sister chromatid exchange formation. Ann Rev Genet 15: 11—15, 1981
46. *Laughlin TJ, Taylor JH:* Initiation of DNA replication in chromosomes of Chinese hamster ovary cells. Chromosoma 75: 19—35, 1979
47. *Lavi S:* Carcinogen-mediated amplification of viral DNA sequences in simian virus 40-transformed Chinese hamster embryo cells. Proc Natl Acad Sci USA 78: 6144—6148, 1981
48. *Lee W-H, Murphree AL, Benedict WF:* Expression and amplification of the N-*myc* gene in primary retinoblastoma. Nature 309: 458—460, 1984
49. *Levan A, Manolov G, Clifford P:* Chromosomes of a human neuroblastoma. A new case with accessory minute chromosomes. J Natl Cancer Inst 41: 1377—1387, 1968
50. *Levan A, Levan G, Mitelman F:* Chromosomes and cancer. Hereditas 86: 15—29, 1977
51. *Lewis JA, Biedler JL, Melera PW:* Gene amplification accompanies low level increases in the activity of dihydrofolate reductase in antifolate-resistant Chinese hamster lung cells containing abnormally banding chromosomes. J Cell Biol 94: 418—424, 1982
52. *Lin CC, Alitalo K, Schwab M, George D, Varmus HE, Bishop JM:* Evolution of karyotypic abnormalities and c-*myc* oncogene amplification in a human colonic carcinoma. Submitted for publication
53. *Little CD, Nau MM, Carney DN, Gazdar AF, Minna JD:* Amplification an expression of the c-*myc* oncogene in human lung cancer cell lines. Nature 306: 194—196, 1983
54. *Lozzio CB, Lozzio BB:* Human chronic myelogenous leukemia cell-line with positive Philadelphia chromosome. Blood 45: 321—334, 1975
55. *Mariani BD, Schimke RT:* Gene amplification in a single cell cycle in Chinese hamster ovary cells. J Biol Chem 259: 1901—1910, 1984
56. *Mark J:* Double minutes-a chromosomal aberration in Rous sarcomas in mice. Hereditas 57: 1—22, 1967
57. *Montgomery KT, Biedler JL, Spengler BA, Melera PW:* Specific DNA sequence amplification in human neuroblastoma cells. Proc Natl Acad Sci USA 80: 5724—5728, 1983
58. *Nowell RC:* The clonal evolution of tumor cell populations. Science 194: 23—28, 1976
59. *Nowell R, Finan J, Favera RD, Gallo RC, Ar-Rushdi A, Romanczuk G, Selden JR, Emanuel BS, Rovera G, Croce CM:* Association of amplified oncogene c-*myc* with an abnormally banded chromosome 8 in a human leukemia cell line. Nature 306: 494—497, 1983
60. *Pall ML:* Gene amplification model of carcinogenesis. Proc Natl Acad Sci USA 78: 2465—2468, 1981
61. *Pelicci P-G, Lanfrancone L, Brathwaite MD, Wolman SR, Dalla-Favera R:* Amplification of the c-*myb* oncogene in a case of human acute myelogenous leukemia. Science 224: 1117—1121, 1984
62. *Pellegrini S, Dailey L, Basilico C:* Amplification and excision of integrated polyoma DNA sequences require a functional origin of replication. Cell. 36: 943—949, 1984
63. *Quinn LA, Moore GE, Morgan RT, Woods LK:* Cell lines from human colon carcinoma with unusual cell products, double minutes, and homogeneously staining regions. Cancer Res 39: 4914, 1979
64. *Rechavi G, Givol D, Canaani E:* Activation of a cellular oncogene by DNA rearrangement: possible involvement of an IS-like element Nature 300: 607—611, 1982
65. *Roberts JM, Buck LB, Axel R:* A structure for amplified DNA. Cell 33: 53—63, 1983
66. *Rowley JD:* Human oncogene locations and chromosome aberrations. Nature 301: 290, 1983
67. *Rowley JD, Testa JR:* Chromosome abnormalities in malignant hematologic diseases. Adv Cancer Res 36: 103—147, 1982
68. *Rowley JD, Ultmann JE eds:* Chromosomes and Cancer: from molecules to man. Academic Press, New York 1983
69. *Saksela K, Bergh J, Lehto V-P, Nilsson K, Alitalo K:* Amplification of the c-*myc* oncogene is characteristic of a subpopulation of human small cell lung cancer. Cancer Res, in press
70. *Schimke RT:* Gene amplification. Cold Spring Harbor Labor 1982
71. *Schimke RT, Brown PC, Kaufman RJ, McGrogan M, Slate DL:* Chromosomal and extrachromosomal localization of amplified dihydrofolate reductase genes in cultured mammalian cells. Cold Spring Harbor Symp Quant Biol 55: 785—797, 1981
72. *Schwab M, Alitalo K, Klempnauer K-H, Varmus HE, Bishop JM, Gelbert F, Brodeur G, Goldstein M, Trent J:* Amplified DNA with limited homology to *myc* cellular oncogne is shared by human neuroblastoma cell lines and a neuroblastoma tumour. Nature 305: 245—248, 1983
73. *Schwab M, Alitalo K, Varmus HE, Bishop JM:* Amplification of cellular oncogenes in tumor cells. In: The cancer cell. Cold Spring Harbor Press 2: 215—220, 1984
74. *Schwab M, Alitalo K, Varmus HE, Bishop JM, George D:* A cellular oncogene (c-Ki-*ras*) is amplified, overexpressed, and located within karyotypic abnormalities in mouse adreocortical tumour cells. Nature 303: 497—501, 1983
75. *Schwab M, Varmus HE, Bishop JM, Grezeschik K-H, Naylor SL, Sakaguchi AY, Brodeur G, Trent J:* Chromosome localization in normal human cells and neuroblastomas of a gene related to c-*myc*. Nature 308: 288—291, 1984
76. *Selden JR, Emanuel BS, Wang E, Cannizzaro L, Palumbo A, Erikson J, Nowell PC, Rovera G, Croce CM:* Amplified C_λ and c-*abl* genes are on the same marker chromosome in K562 leukemia cells. Proc Natl Acad Sci USA 80: 7289—7292, 1983
77. *Shimizu K, Goldfarb M, Perucho M, Wigler M:* Isolation and preliminary characterization of the transforming gene of human neuroblastoma cell line. Proc Natl Acad Sci USA 80: 383—387, 1983
78. *Shmookler Reis RJ, Lumpkin CK, McGill JR, Riabowol TK, Goldstein S:* Extrachromosomal circular copies of an 'inter-Alu' unstable sequence in human DNA are amplified during in vitro and in vivo ageing. Nature 201: 394—398, 1983
79. *Stark GR, Wahl GM:* Gene amplification. Ann Rev Biochem 53: 447—491, 1984
80. *Taya Y, Hesogai K, Hirohashi S, Shimosato Y, Tsuchiya R, Tsuchida N, Fushimi M, Sekiya T, Nishimura S:* A novel combination of K-*ras* and *myc* amplification accompanied by point mutational activation of K-*ras* in human lung cancer. EMBO J 3: 2943—2946, 1984
81. *Tlsty TD, Brown PC, Schimke TR:* UV radiation facilitates methotrexate resistance and amplification of the dihydrofolate reductase gene in cultured 3T6 mouse cells. Molec Cell Biol 4: 1050—1056, 1984

82. *Ullrich A, Coussens L, Hayklick JS, Dull TJ, Gray A, Tam AW, Lee J, Yarden Y, Libermann TA, Sschlessinger J, Downward J, Mayes ELV, Whittle N, Waterfield MD, Seeburg PH:* Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermoid carcinoma cells. Nature 309: 418—425, 1984
83. *Wahl GM, de Saint Vincent BR, DeRose ML:* Effect of chromosomal position on amplification of transfected genes in animal cells. Nature 307: 516—520, 1984
84. *Varshavsky A:* On the possibility of metabolic control of replicon 'misfiring': Relationship to emergence of malignant phenotypes in mammalian cell lineages. Proc Natl Acad Sci USA 78: 3673—3677, 1981
85. *Varshavsky A:* Phorbol ester dramatically increases incidence of methotrexate-resistant mouse cells: Possible mechanisms and relevance to tumor promotion. Cell 25: 561—572, 1981
86. *Whang-Peng J, Bunn PA, Kao-Shan CS, Lee EC, Carney DN, Gazdar A, Minna JD:* A Nonrandom chromosomal abnormality, del 3p (14—23), in human small cell lung cancer (SCLC). Cancer Genet Cytogenet 6: 119—134, 1982
87. *Whang-Peng J, Kao-Shan CS, Lee EC, Bunn PA, Carney DN, Gazdar AF, Portlock C, Minna JD:* Deletion 3p (14—23), Double minute chromosomes, and homogeneously staining regions in human small-cell lung cancer. sCSH Laboratory 1982
88. *Winqvist R, Knuutila S, Leprince D, Stehelin D, Alitalo K:* Mapping of amplified c-*myb* oncogene, sister chromatid exchanges and karyotypic analysis of the COLO 205 colon carcinoma cell line. Cancer Genet Cytogenet. in press
89. *Winqvist R, Saksela K, Alitalo K: myc* proteins are not associated with chromatin in mitotic cells. EMBO J 3: 2947—2950
90. *Woodcock DM, Cooper IA:* Evidence for double replication of chromosomal DNA segments as a general consequence of DNA replication inhibition. Cancer Res 41: 2483—2490, 1981
91. *Zabel BU, Naylor SL, Grezeschik K-H, Sakaguchi AY:* Regional assignment of human protooncogene c-*myb* to 6q21—qter. Somatic Cell Molec Genet 10: 105—108, 1984

Received for publication: September 19, 1984
Address: K. Alitalo
Department of Virology
University of Helsinki
SF-00290 Helsinki
Finland

Elevated Epidermal Growth Factor Receptor Gene Copy Number and Expression in a Squamous Carcinoma Cell Line

Glenn T. Merlino, Young-hua Xu, Nancy Richert, Adrian J. L. Clark, Shunsuke Ishii, Susan Banks-Schlegel, and Ira Pastan

Laboratory of Molecular Biology, Division of Cancer Biology and Diagnosis, and Laboratory of Human Carcinogenesis, Division of Cancer Etiology, National Cancer Institute, National Institutes of Health, Bethesda, Maryland 20205

Abstract

The human epidermal growth factor (EGF) receptor is known to be homologous to the v-*erb* B oncogene protein of the avian erythroblastosis virus. Overexpression of the EGF receptor gene in A431 epidermoid carcinoma cells is due to gene amplification. In this study, a variety of squamous cell carcinomas were examined and one, SCC-15, contained high levels of the EGF receptor as determined by immunoprecipitation via an EGF receptor-specific polyclonal antibody. Using a cloned EGF receptor complementary DNA as a probe, the level of EGF receptor RNA was found to be elevated four-fold in SCC-15 relative to normal cultured keratinocytes. When the same probe was used to identify EGF receptor gene fragments on a genomic DNA blot, the SCC-15 cell line was shown to possess an EGF receptor gene copy number amplified four to five times. Gene amplification results in the enhancement in the level of the EGF receptor in several carcinomas and could be responsible for the appearance of the transformed phenotype in these cells.

Introduction

The epidermal growth factor (EGF)[1] stimulates growth and elicits a wide variety of rapid and delayed responses by binding to high-affinity cell-surface receptors which are 170-kD glycoproteins (1). Recently, EGF receptor peptides have been sequenced and found to be homologous to the avian erythroblastosis virus *erb* B oncogene product (2), suggesting that the EGF receptor gene is the human c-*erb* B oncogene. A431 epidermoid carcinoma cells possess a very large number of EGF receptors (3), and the EGF receptor gene is amplified ~30-fold (4–6). This amplification is responsible for the overexpression of the EGF receptor protein in these cells (4–6).

A cell culture system has been developed permitting serial cultivation of keratinocytes, whose growth is modulated by EGF (7). Such methods have been used to establish cell lines from squamous cell carcinomas of the oral epithelium (8). Because of the role of EGF in keratinocyte development, we quantified EGF receptor protein and RNA in several squamous cell carcinomas. One cell line, SCC-15, was found to contain high amounts of receptor protein and RNA, and a four- to fivefold amplification of the gene.

Methods

The squamous cell carcinomas established by Rheinwald and Beckett (8) were obtained from, and maintained according to the American Type Culture Collection (Rockville, MD). 1623 was originally designated as SCC-15; 1628 as SCC-25; and 1629 as SCC-9 (8). Normal human esophageal epithelial cells were grown as reported (9). Maintenance of other cell lines was as described elsewhere (10). Proteins were labeled with [^{35}S]methionine and immunoprecipitated as previously described (10). PolyA+ RNA was isolated by guanidine isothiocyanate solubilization and CsCl centrifugation (11), and oligo(dT)-affinity chromatography. RNA (Northern) blotting was performed as described (11, 12). High molecular weight DNA was isolated (4) and analyzed by DNA (Southern) blotting (4, 10, 13). The EGF receptor complementary DNA (cDNA) clone pE7 was constructed and isolated from an A431 cDNA library (11). DNA fragments were ^{32}P-labeled by nick translation.

Results

A large number of cell lines were initially screened for EGF receptor levels by determining their ability to be killed by an EGF-pseudomonas exotoxin conjugate, a technique described previously (10). Several squamous cell carcinomas were found to be relatively sensitive to the EGF-toxin conjugate, including SCC-25, SCC-9, and particularly SCC-15, all derived from the human tongue (8). These three cell lines were labeled with [^{35}S]methionine, and their extracts immunoprecipitated with a goat polyclonal antibody to the EGF receptor, affinity-purified as described (10). When compared with A431 cells, which make very large amounts of the EGF receptor, SCC-25 and SCC-9 make moderate amounts and SCC-15 high amounts of the receptor (Fig. 1, lane *a* vs. *e*, *g*, and *c*). Quantitation of the immunoprecipitation data revealed that SCC-15, SCC-25, and SCC-9 make 41, 15, and 4% of the amount of EGF receptor made by A431 cells, respectively.

Because SCC-15 cells had high levels of receptor, polyA+ RNA was isolated from these cells, electrophoretically fractionated on agarose, and analyzed by RNA (Northern) blotting. A cloned A431 cDNA (pE7) encoding the EGF receptor (11) was ^{32}P-labeled and used as a hybridization probe to visualize EGF receptor RNAs. Fig. 2 *A* shows that SCC-15 contains both the 10- and 5.6-kilobase species of EGF receptor RNA. The levels are approximately four- to fivefold higher than those found in either KB or A498 kidney carcinoma cells; these cell lines were previously found to possess readily de-

Received for publication 21 November 1984.

1. *Abbreviations used in this paper:* cDNA, complementary DNA; EGF, epidermal growth factor.

The Journal of Clinical Investigation, Inc.
Volume 75, March 1985, 1077–1079

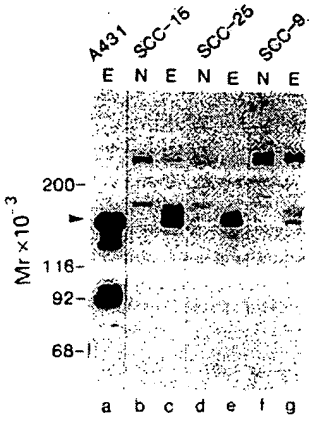


Figure 1. Autoradiograph of electrophoresed ^{35}S-labeled EGF receptor protein (arrow) immunoprecipitated by either E, affinity-purified goat polyclonal EGF receptor antibody, or N, normal serum. Molecular weight markers are at left.

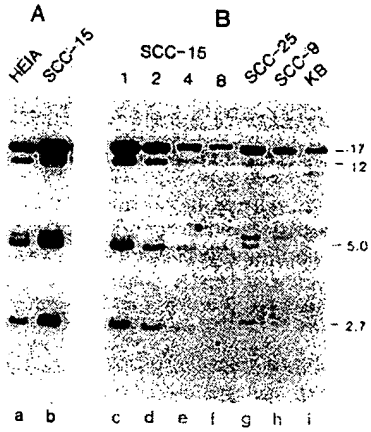


Figure 3. DNA blot analysis of *Hin*dIII-digested genomic DNAs using the pE7 probe (see Fig. 2). (*A*) and (*B*) are autoradiographs from two separate gels. Sizes are in kilobase pairs (right). 10 μg of DNA was loaded except in (*B*), lanes *d–f*, which represent serial dilutions of the 1623 DNA shown in lane *c*.

tectable levels of both receptor RNAs (10). Fig. 2 *B* shows that cultured human epithelial cells contain EGF receptor-specific RNA (HEIA, lane *e*) whose levels are higher than an early passage human fibroblast D551 (lane *f*), equivalent to A498 (lane *g*), but much lower than SCC-15 (lane *b*).

To determine if an elevated gene copy number was associated with enhanced expression of the EGF receptor gene in SCC-15 cells, genomic DNA was isolated from normal cultured epithelial cells (HEIA) and SCC-15 cells, digested with *Hin*dIII, electrophoretically fractionated, and subjected to DNA blotting analysis. An EGF receptor cDNA (pE7) was used as a hybridization probe to identify receptor DNA fragments. Fig. 3 *A* reveals that the SCC-15 genome contains four- to fivefold amplified EGF receptor gene sequences relative to normal epithelial cells (lane *a* vs. *b*). Analysis of β-actin gene fragments on the same filter by hybridization to a chick actin cDNA probe indicated that equal amounts of DNA were loaded per well (data not shown). Digested SCC-15 DNA had to be diluted about fourfold (Fig. 3 *B*, lane *e*) to approximate the signal intensity of receptor DNA fragments from SCC-25, SCC-9, and KB cells (lanes *g–i*). The KB cell EGF receptor gene is known not to be amplified (10).

Discussion

It may be significant that A431 carcinoma cells are not unique in their possession of amplified EGF receptor genes. We report here that the EGF receptor gene in squamous cell carcinoma SCC-15 is amplified four- to fivefold relative to normal epithelial cells. The amplification of the EGF receptor gene may cause the initiation or maintenance of the malignant state in some human cells.

Previously, we reported that a variety of transformed cell lines synthesize relatively high amounts of both EGF receptor protein and messenger RNA (10). It is conceivable that a moderate or even a small increase in the level of the EGF receptor leads to a change in the cellular phenotype, as has been demonstrated for the *src* gene product (14). If this hypothesis is correct, then even a minor amplification of the EGF receptor gene copy number could contribute to the onset of tumorigenesis. Hendler and Ozanne (15) have examined lung squamous cell carcinomas and found that they contain a 2.5–5-fold increase in EGF receptor levels.

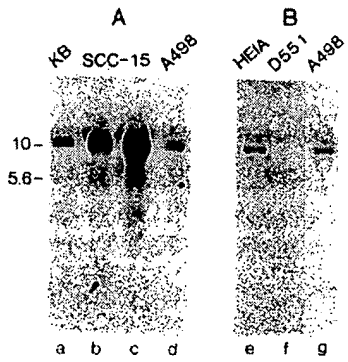


Figure 2. RNA blot analysis of polyA+ RNAs using the ^{32}P-labeled EGF receptor cDNA probe pE7. (*A*) and (*B*) are autoradiographs from two separate gels. Sizes are in kilobases (left). 5 (lanes *a*, *b*, *d*, and *g*) or 10 (lanes *c*, *e*, and *f*) μg of RNA were loaded.

References

1. Savage, C. R., and S. J. Cohen. 1972. Epidermal growth factor and a new derivation: rapid isolation procedure and biological and chemical characterization. *J. Biol. Chem.* 247:7609–7611.

2. Downward, J., Y. Yarden, E. Mayes, G. Scarce, N. Totty, P. Stockwell, A. Ullrich, J. Schlessinger, and M. D. Waterfield. 1984. Close similarity of the epidermal growth factor receptor and v-erb B oncogene protein sequence. *Nature (Lond.).* 307:521–527.

3. Fabricant, R. N., J. E. DeLarco, and G. J. Todaro. 1977. Nerve growth factor receptor on human melanoma cells in culture. *Proc. Natl. Acad. Sci. USA.* 74:565–569.

4. Merlino, G. T., Y.-H. Xu, S. Ishii, A. J. L. Clark, K. Semba, K. Toyoshima, T. Yamamoto, and I. Pastan. 1984. Amplification and enhanced expression of the epidermal growth factor receptor gene in A431 human carcinoma cells. *Science (Wash. DC).* 224:417–419.

5. Ullrich, A., L. Coussens, J. S. Hayflick, T. J. Dull, A. Gray, A. W. Tam, J. Lee, Y. Yarden, T. A. Libermann, J. Schlessinger, J. Downward, E. L. V. Mayes, N. Whittle, M. D. Waterfield, and P. H. Seeburg. 1984. Human epidermal growth factor receptor cDNA sequence and aberrant expression of the amplified gene in A431 epidermal carcinoma cells. *Nature (Lond.).* 309:418–425.

6. Lin, C. R., W. S. Chen, W. Kruiger, L. S. Stolarsky, W. Weber, R. M. Evans, I. M. Verma, G. N. Gill, and M. G. Rosenfeld. 1984.

Expression cloning of human EGF receptor complementary DNA: gene amplification and three related messenger RNA products in A431 cells. *Science (Wash. DC).* 224:843–848.

7. Rheinwald, J. G., and H. Green. 1975. Serial cultivation of strains of human epidermal keratinocytes: the formation of keratinizing colonies from single cells. *Cell.* 6:331–344.

8. Rheinwald, J. G., and M. A. Beckett. 1981. Tumorigenic keratinocyte lines requiring anchorage and fibroblast support cultured from human squamous cell carcinomas. *Cancer Res.* 41:1657–1663.

9. Banks-Schlegel, S. P., and C. C. Harris. 1983. Tissue-specific expression of keratin proteins in human esophageal and epidermal epithelium and their cultured keratinocytes. *Exp. Cell Res.* 146:271–280.

10. Xu, Y.-H., N. Richert, S. Ito, G. T. Merlino, and I. Pastan. 1984. Characterization of epidermal growth factor receptor gene expression in malignant and normal human cell lines. *Proc. Natl. Acad. Sci. USA.* 81:7308–7312.

11. Xu, Y.-H., I. Ishii, A. J. L. Clark, M. Sullivan, R. K. Wilson, D. P. Ma, B. A. Roe, G. T. Merlino, and I. Pastan. 1984. Human epidermal growth factor receptor cDNA is homologous to a variety of RNAs overproduced in A431 carcinoma cells. *Nature (Lond.).* 309: 806–810.

12. Thomas, P. S. 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. *Proc. Natl. Acad. Sci. USA.* 77:5201–5205.

13. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503–517.

14. Jakobovits, E. B., J. E. Majors, and H. E. Varmus. 1984. Hormonal regulation of the Rous sarcoma src gene via a heterologous promoter defines a threshold dose for cellular transformation. *Cell.* 38: 757–765.

15. Hendler, F. J., and B. W. Ozanne. 1984. Human squamous cell lung cancers express increased epidermal growth factor receptors. *J. Clin. Invest.* 74:647–651.

BMC Gastroenterology

BioMed Central

Research article

Open Access

Cyclin A and cyclin D1 as significant prognostic markers in colorectal cancer patients

Abeer A Bahnassy*[1], Abdel-Rahman N Zekri[2], Soumaya El-Houssini[1], Amal MR El-Shehaby[3], Moustafa Raafat Mahmoud[1], Samira Abdallah[4] and Mostafa El-Serafi[5]

Address: [1]Pathology Department, National Cancer Institute, Cairo University, Cairo, Egypt, [2]Virology and Immunology Unit, Cancer Biology Department, National Cancer Institute, Cairo University, Cairo, Egypt, [3]Biochemistry Department, Kasr El-Eini School of Medicine, Cairo University, Cairo, Egypt, [4]Pathologyy Department, Kasr El-Eini School of Medicine, Cairo University, Cairo, Egypt and [5]Medical Oncology Department, National Cancer Institute, Cairo University, Cairo, Egypt

Email: Abeer A Bahnassy* - chaya2000@hotmail.com; Abdel-Rahman N Zekri - ncizakri@starnet.com.eg; Soumaya El-Houssini - chaya2000@hotmail.com; Amal MR El-Shehaby - chaya2000@hotmail.com; Moustafa Raafat Mahmoud - ncizakri@starnet.com.eg; Samira Abdallah - chaya2000@hotmail.com; Mostafa El-Serafi - melserafi@starnet.com.eg

* Corresponding author

Published: 23 September 2004

BMC Gastroenterology 2004, 4:22 doi:10.1186/1471-230X-4-22

Received: 25 April 2004

Accepted: 23 September 2004

This article is available from: http://www.biomedcentral.com/1471-230X/4/22

Abstract

Background: Colorectal cancer is a common cancer all over the world. Aberrations in the cell cycle checkpoints have been shown to be of prognostic significance in colorectal cancer.

Methods: The expression of *cyclin D1*, *cyclin A*, *histone H3* and *Ki-67* was examined in 60 colorectal cancer cases for co-regulation and impact on overall survival using immunohistochemistry, southern blot and in situ hybridization techniques. Immunoreactivity was evaluated semi quantitatively by determining the staining index of the studied proteins.

Results: There was a significant correlation between *cyclin D1* gene amplification and protein overexpression (concordance = 63.6%) and between *Ki-67* and the other studied proteins. The staining index for *Ki-67, cyclin A and D1* was higher in large, poorly differentiated tumors. The staining index of *cyclin D1* was significantly higher in cases with deeply invasive tumors and nodal metastasis. Overexpression of *cyclin A* and *D1* and amplification of *cyclin D1* were associated with reduced overall survival. Multivariate analysis shows that *cyclin D1 and A* are two independent prognostic factors in colorectal cancer patients.

Conclusions: Loss of cell cycle checkpoints control is common in colorectal cancer. *Cyclin A and D1* are superior independent indicators of poor prognosis in colorectal cancer patients. Therefore, they may help in predicting the clinical outcome of those patients on an individual basis and could be considered important therapeutic targets.

Background

Colorectal cancer (CRC) is the third most common cancer in Western countries [1]. In Egypt, CRC has unique characteristics that differ from that reported in other countries of the western society. It was estimated that 35.6% of the Egyptian CRC cases are below 40 years of age and patients

usually present with advanced stage, high grade tumors that carry more mutations [2]. This uniquely high proportion of early-onset CRC, the early and continuous exposure to hazardous environmental agents, the different mutational spectrum and the prevalent consanguinity in Egypt justify further studies [3]. It was proved that most cancers result from accumulation of genetic alterations involving certain groups of genes, the majority of which are cell cycle regulators that either stimulate or inhibit cell cycle progression [1]. Cell proliferation allows orderly progression through the cell cycle, which is governed by a number of proteins including *cyclins* and *cyclin* dependent kinases [4,5]. The *cyclins* belong to a superfamily of genes whose products complex with various *cyclin*-dependent kinases (*cdks*) to regulate transitions through key checkpoints of the cell cycle [6]. Abnormalities of several *cyclins* have been reported in different tumor types, implicating, in particular, *cyclin A*, *cyclin E* and *cyclin D* [6,7].

Cyclin D1 is a G1 *cyclin* that regulates the transition from G1 to S phase since its peak level and maximum activity are reached during the G1 phase of the cell cycle. Whereas *cyclin A* is regarded a regulator of the transition to mitosis since it reaches its maximum level during the S and G2 phases [8]. The mechanisms likely to activate the oncogenic properties of the *cyclins* include chromosomal translocations, gene amplification and aberrant protein overexpression [7,9].

Several studies have shown that, *histone H3* mRNA expression can be used to identify the S phase fraction (SPF) through the in situ hybridization (ISH) technique [10,11]. The level of *histone H3* mRNA reaches its peak during the S phase and then drops rapidly at the G2 phase [12].

In face of the increasing incidence of CRC and its peculiar pattern in the Egyptian population, the present study was conducted to assess the role of *Ki-67* (pan-cell cycle marker), *cyclin D1* (G1 phase marker), *histone H3* mRNA (S phase marker), *cyclin A* (S to G2 phase marker) in CRC. The expression level of these markers was correlated to the clinicopathologic features and the overall survival of patients.

Table 1: Clinicopathological features of patients in relation to the staining index (SI) of *Ki-67*, *cyclin D1*, *cyclin A*, *histone H3*

		SI (mean + SD)			
Variables	*No. of cases*	*Ki-67*	*Cyclin DI*	*Cyclin A*	*Histone H3*
Sex					
Male	36	18.0 ± 6.4	6.7 ± 4.3	12.7 ± 5.7	10.7 ± 5.3
Female	24	20.1 ± 5.8	8.8 ± 8.4	10.0 ± 6.0	10.7 ± 5.4
Age (years)					
≥50	41	11.7 ± 6.0*	5.6 ± 5.2	10.0 ± 5.3	6.0 ± 5.0*
<50	19	23.8 ± 5.6	7.7 ± 6.8	13.6 ± 5.7	22.0 ± 5.2
Tumor size (cm)					
<5.0	33	12.2 ± 6.3*	5.3 ± 3.8*	11.5 ± 6.1*	10.3 ± 4.9*
≥5.0	27	30.1 ± 6.2	22.8 ± 7.2	28.6 ± 5.6	24.0 ± 5.6
Histology					
Normal	20	3.5 ± 2.0*	0.6 ± 0.2*	2.3 ± 1.1*	2.2 ± 0.9
Carcinoma	60	30.3 ± 6.2	24.9 ± 6.3	27.2 ± 5.8	10.7 ± 5.3
GI	15	11.7 ± 6.2	6.6 ± 4.0	10.0 ± 5.4	11.4 ± 4.9
GII	21	11.8 ± 5.6	8.9 ± 3.6	12.3 ± 6.5	7.8 ± 5.4
GIII	24	30.0 ± 4.3	22.0 ± 8.1	27.0 ± 4.9	11.5 ± 5.4
Lymph node					
Negative	33	19.5 ± 7.0	5.4 ± 5.3*	11.9 ± 6.5	12.3 ± 5.5
Positive	27	21.3 ± 4.9	20.6 ± 6.9	12.5 ± 5.0	14.2 ± 5.0
Depth of invasion					
m, sm	17	20.7 ± 6.7	3.1 ± 3.1*	11.9 ± 7.2	10.4 ± 5.1
beyond sm	43	21.9 ± 6.2	12.4 ± 6.5	12.2 ± 5.6	10.7 ± 5.4
Stage					
I	6	20.6 ± 6.7	5.7 ± 6.9	24.2 ± 6.9	11.1 ± 5.3
II	27	20.8 ± 6.9	5.3 ± 4.3	24.6 ± 6.0	10.4 ± 5.7
III	12	22.0 ± 5.4	7.7 ± 6.0	27.1 ± 5.2	10.4 ± 4.9
IV	15	24.7 ± 6.1	11.3 ± 9.6	27.5 ± 5.5	12.3 ± 6.2

* p. value < 0.05 (significant)

Methods

Tissue samples

Paraffin-embedded tumor tissues were obtained from 60 CRC patients (47 colon and 13 rectal carcinomas) that were diagnosed and treated at the National Cancer Institute, Cairo, Egypt during the period from January, 1997 to June, 2002. Clinicopathological data of the studied cases are illustrated in table 1. None of the patients received any chemotherapy or irradiation prior to surgery. Histological diagnosis of all cases was done by 2 independent pathologists according to the WHO Histological Classification. Tumors were staged according to the TNM staging system [13]. The depth of tumor invasion was classified as invasion of the mucosa including muscularis mucosa (m), invasion of the submucosa (sm), or invasion beyond the submucosa [8]. Normal colonic tissues were obtained from autopsy specimens (n = 20) and were used as a control. The actual survival rate of the patients was calculated from the date of resection to the date of death.

Immunohistochemistry

Four micron sections of each normal and tumor specimen were cut onto positive-charged slides; air dried overnight, de-paraffinized in xylene, hydrated through a series of graded alcohol and washed in distilled water and 0.01 PBS (pH 7.4). Slides were then processed for IHC as described by Handa et al. [8]. using the following antibodies: *Ki-67* (MIB-1, Dako), *cyclin A* (6E6; Novocastra, Newcastle-Upon-Tyne, UK) and *cyclin D1* (DCS-6, Dako). A case of invasive breast cancer was used as a positive control for *Ki-67* and *cyclin A* whereas a case of mantle cell lymphoma was used as a control for *cyclin D1*. Negative controls were obtained by replacing the primary antibody by non-immunized rabbit or mouse serum.

Brown nuclear staining was regarded as a positive result for all studied markers. The proportion of positively-stained cells and the intensity of staining were scored in tumor and normal colorectal mucosal sections at medium power (×200). The degree of positive tumor staining (percentage of positive tumor cells in the examined section) was scored from 1–6 and the staining intensity was scored from 0–6 according to the pattern of staining in the examined section. Staining index (SI) was calculated by multiplying the cellularity and staining scores as described by King et al. [14].

In situ hybridization

All tumor samples and 5 normal controls were assessed for *histone H3* mRNA by ISH using the commercially available 550 base fluorescein-labeled DNA probe (Dako, Carpinteria, CA) as described by Nagao et al., 1996. This probe hybridizes to the whole mRNA transcript of the human *histoneH3* gene including the5' and 3' un-translated regions. Scoring of *histone H3* mRNA was performed as for immunohistochemistry, however, hybridization signals were detected in the cytoplasm.

Molecular detection of cyclin D1 gene amplification

High molecular weight DNA was extracted from paraffin-embedded tissues of the tumor and normal colorectal mucosal samples as previously described [15]. The proportion of neoplastic and normal cells was determined in each tumor sample by examining hematoxylin and eosin-stained slides obtained from the edge of the specimen used for DNA extraction. Tumor samples were evaluated for amplification of *cyclin D1* if more than 75% of the examined sections were formed of neoplastic cells. Accordingly, 50 cases were eligible for the analysis. Ten micrograms of the extracted DNA was digested with *Eco*R1. DNA from selected cases was also digested with *Bgl*II and *Hind*III. Samples were separated on 0.8% agarose gels and transferred to Hybond-N membranes (Amersham Int., Amersham, UK). The membranes were hybridized with 50% formamide, 5 × SSC, 5 × Denhardt's, 500 μg/ml denatured salmon sperm DNA, 10% dextran sulphate and 10^6 cpm/ml of ^{32}P-labeled *PRAD-1* probe for 24 h. Membranes were washed with 2 × SSC, 0.1% SDS at room temperature for 30 min followed by 2 × SSC, 0.1% SDS at 60°C for 30 min and 0.1 × SSC, 0.1% SDS at 60°C for 1 h. Filters were autoradiographed using an intensifying screen at -70°C for 24–72 h. After being stripped free of the *PRAD-1* probe, the same blots were hybridized with ^{32}P-labeled *B-actin* probe to normalize against possible variations in the loading or transfer of DNA. The autoradiograms were analyzed using a densitometer. Intensities of *PRAD-1/cyclin D1* were normalized to the *β-actin* control bands. The degree of amplification was calculated from these normalized values. Amplification was considered when the signal of the tumor band was ≥2-fold the value of the matched normal mucosa [16].

Statistical analysis

The Mann-Whitney non-parametric test was used to compare the SIs of pairs of subjects whereas the Kruskal-wallis was used for categorial data. Correlation between indices was performed using a simple linear regression test. The Kaplan-Meier method was used to create survival curves which were analyzed by the log-rank test. The impact of different variables on survival was determined using the Cox proportional hazards model. *p*. values less than 0.05 were considered significant.

Results

The results of IHC are illustrated in figures 1 and 2. In general, the staining index (SIs) of all studied markers was higher in carcinomas than in normal colonic mucosal samples ($p = 0.0001$). Normal colorectal mucosa revealed positive imunostaining for *Ki-67* in the lower half of the crypts only. A heterogeneous staining pattern was

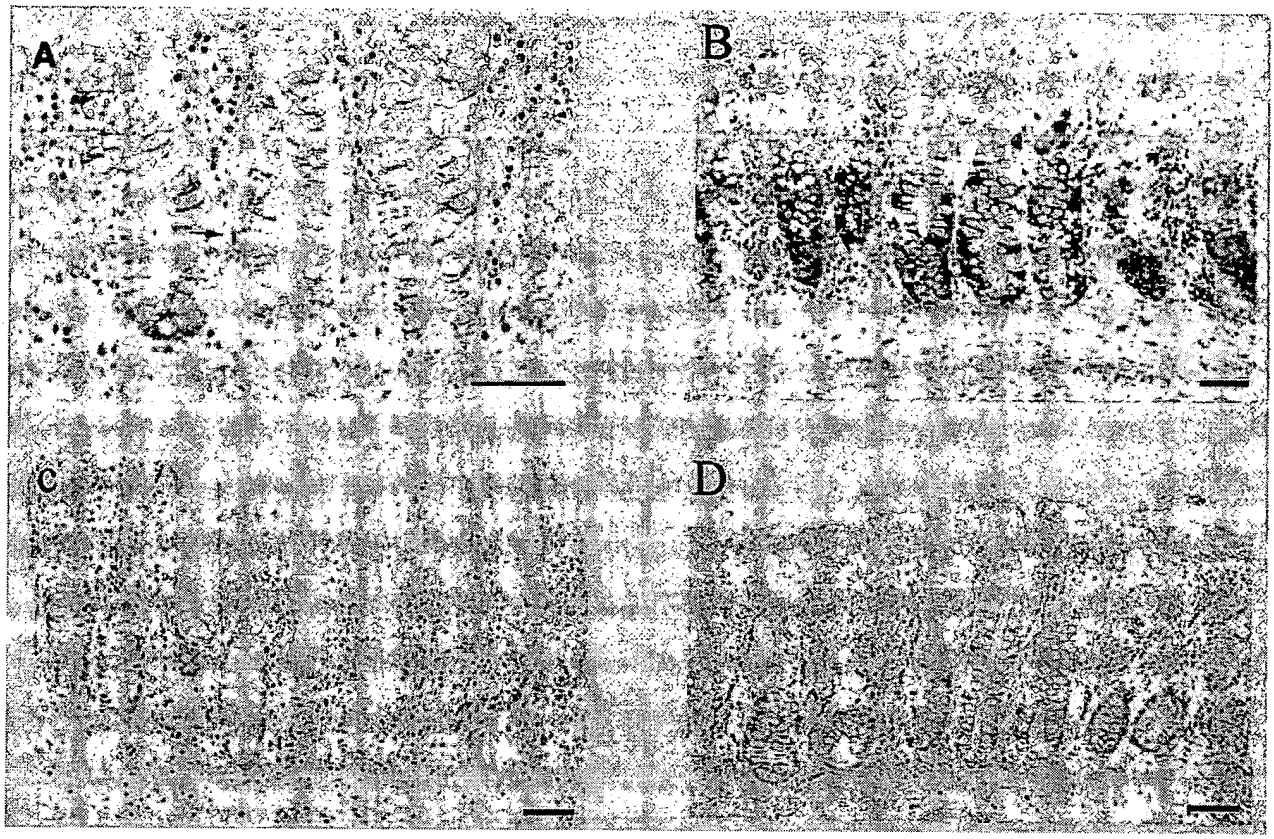


Figure 1
Normal colonic mucosa showing positive nuclear immunostaining for: (a) *cyclin D1*, (b) ISH of *histone H3* mRNA, (c) *Ki-67* and (d) *cyclin A*

detected in the neoplastic cells of well and moderately-differentiated adenocarcinomas whereas a diffuse homogeneous staining pattern was detected in poorly-differentiated carcinomas. The SI ranged from 10–40.2 (mean: 24.6 ± 6.5).

Immunostaining for *cyclin D1* was predominantly nuclear but cytoplasmic staining was detected in some cases. However, unless a nuclear staining was also detected, cases with cytoplasmic staining were considered negative. Normal colorectal mucosal samples were almost negative for *cyclin D1* whereas 41 out of the 60 (68.3%) CRC cases were positive. Marked heterogeneity was observed in well- and moderately-differentiated adenocarcinomas even within the same tumor. Poorly-differentiated carcinomas revealed a diffuse staining pattern with more darkly-stained nuclei. The SI ranged from 0.5–28.6 (mean: 9.3 ± 4.2).

Positive nuclear staining for *cyclin A* was detected in 80% (48/60) of CRC cases and in all non-neoplastic control samples. Positively-stained nuclei were confined to the lower half of the crypts in normal colonic mucosa and diffusely-dispersed in carcinomas. The SI ranged from 3.3–30.2 (mean: 15.1 ± 6.6).

Histone H3 mRNA was intensely expressed in the cytoplasm of all examined samples either neoplastic or non-neoplastic. The distribution of *histone H3* mRNA was similar to that of *cyclin A* and *Ki-67* however, the proportion of *histone H3* mRNA positive cells was less than that of *Ki-67*. The SI ranged from 1.8–24.2 (mean: 12.4 ± 5.3).

The *PRAD-1* probe detected 3 *Eco*RI fragments of 4.0, 2.2 and 2.0 and 1 *Bgl*II fragment of 15 Kb. *PRAD-1/cyclin D1* gene amplification was detected in 22/50 (44%) cases analyzed. The degree of amplification was heterogeneous

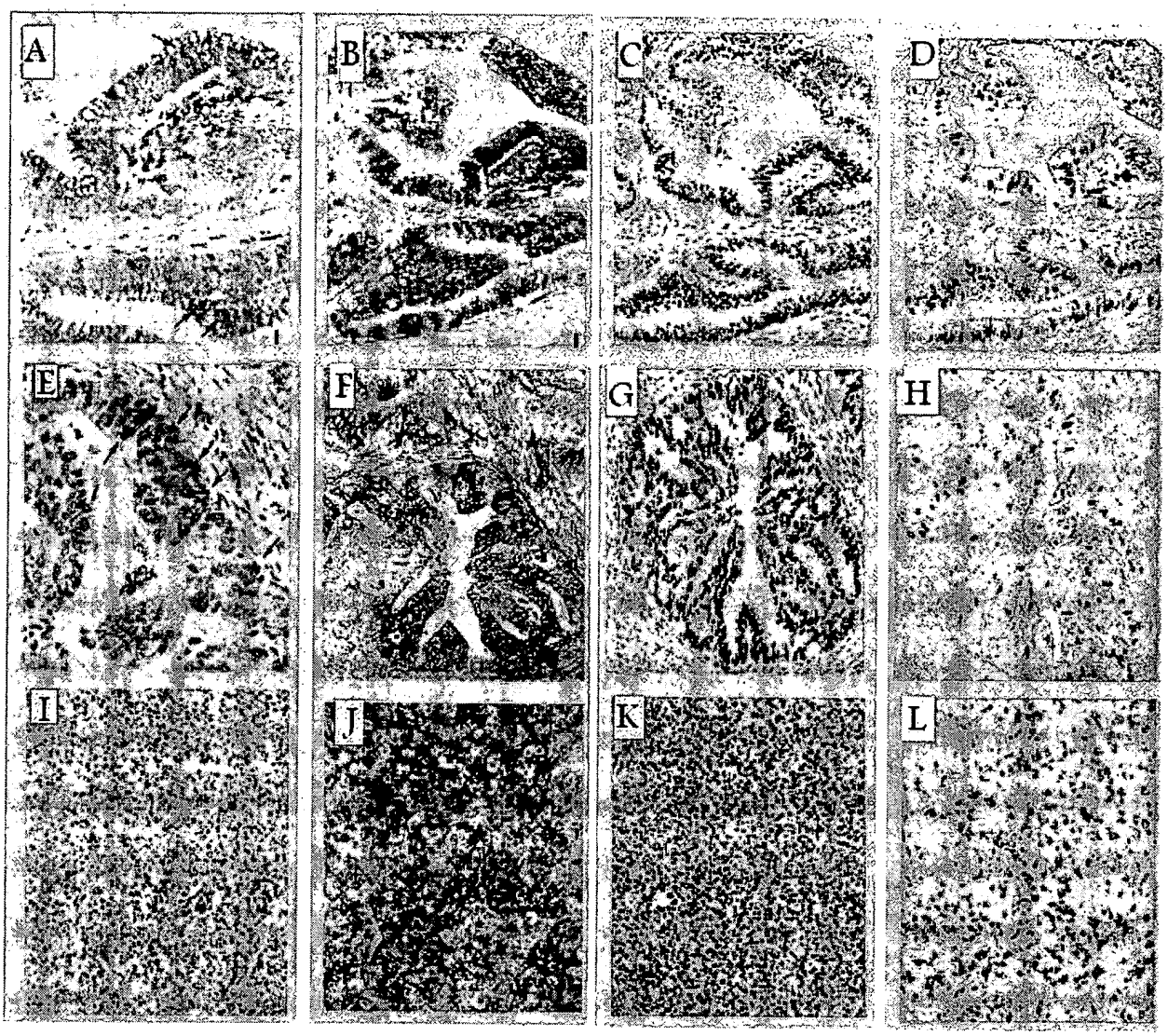


Figure 2
A case of well differentiated adenocarcinoma with positive immunostaining for: (a) *cyclin D1*, (b) *histone H3* mRNA, (c) *Ki-67*, and (d) *cyclin A*. Another case of moderately differentiated denocarcinoma with positive immunostaining for: (e) cyclin D1, (f) *histone H3* mRNA, (g) *Ki-67*, and (h) *cyclin A*. A case of poorly differentiated adenocarcinoma with diffuse staining for: (i) *cyclin D1*, (j) ISH of *histone H3* mRNA, (k) *Ki-67* and (l) *cyclin A*.

with 2–10 fold increase when compared to normal mucosal samples (Figure 3). Amplification was confirmed by other restriction enzymes.

Correlations

There was a significant correlation between *cyclin D1* gene amplification and protein overexpression. Out of the 22 cases that showed amplification 14 showed protein overexpression (concordance = 63.6%).

Linear regression analysis of SIs revealed a significant correlation between *Ki-67* and *cyclin D1*, *cyclin A*, *histone H3* as well as between the SIs of *cyclin A* and *histone H3* (p = 0.008, 0.0001, and 0.0001 respectively) (Figure 4). There was a significant relationship between the SI of both *Ki-67*

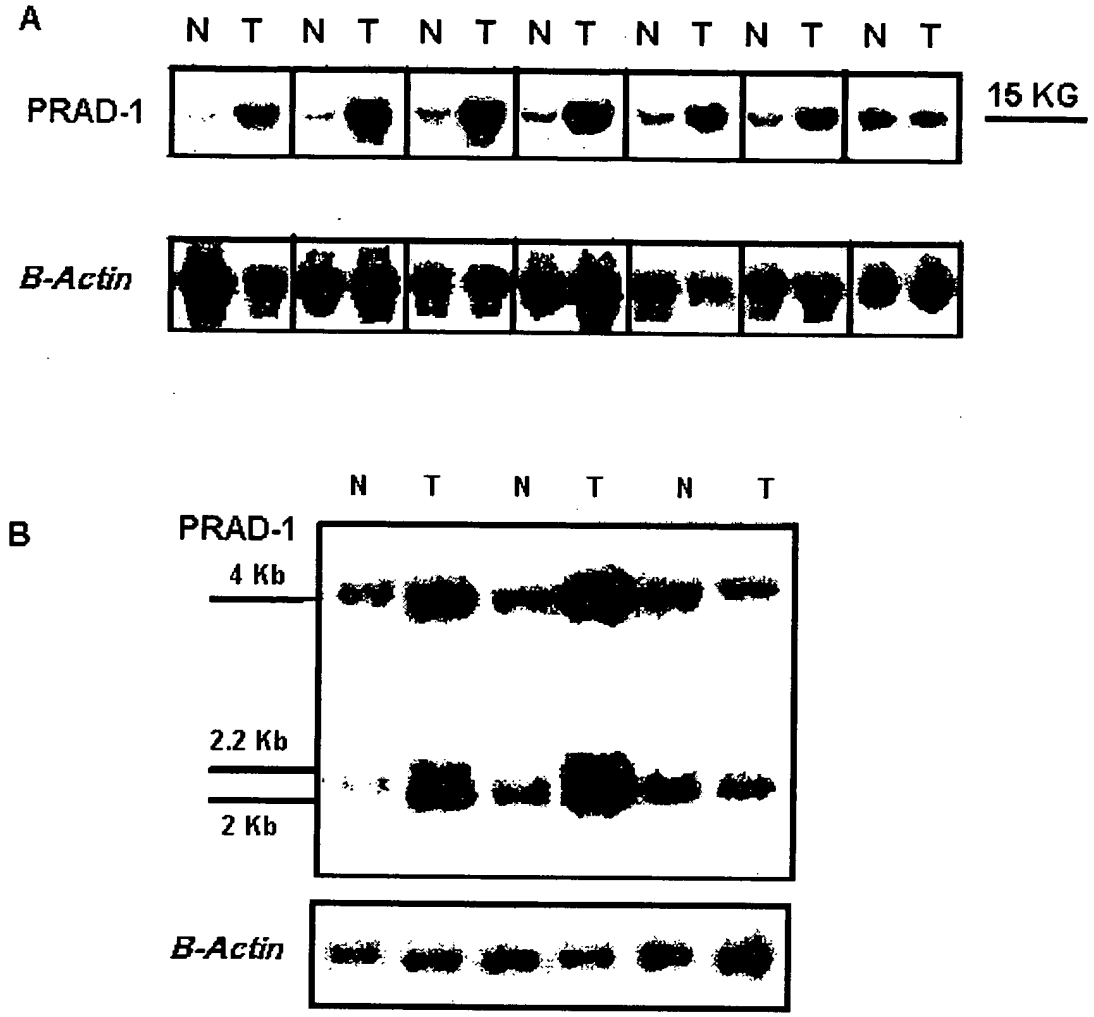


Figure 3

A: Southern blot analysis of normal mucosa (N) and their seven corresponding cases of colonic adenocarcinomas (T1–T7), cases No. 1, 2, 4, and 5 are poorly differentiated whereas cases No. 3, 6, and 7 are moderately differentiated. Genomic DNA was digested with *BglII*, fractionated by electrophoresis in agarose gel, transferred onto membranes and hybridized with *PRAD1* and *β-actin*. Tumors number 1–6 (Lanes 1–6) show different degrees of *PRAD1/cyclin D1* amplification, tumor number 7 (lane 7) was not amplified. **B:** Southern blot analysis of 3 cases of adenocarcinomas (T) and matched normal colonic mucosa (N). Genomic DNA was digested with *EcoRI*, fractionated by electrophoresis in agarose gel, transferred onto membranes and hybridized with *PRAD1* and *β-actin* probes for loading control. The identification of the 3 tumors is the same as in Fig. 3A with amplification of *PRAD1/cyclin D1* in tumors number 4, 5 (Lanes 1, 2) but not 7 (Lane 3).

and *cyclin A* and the degree of differentiation of tumors as well as the size of the tumor ($p < 0.001$ and $p < 0.01$ respectively). In addition, SI of *Ki-67* and *histone H3* were higher in patients <50 years than in those ≥50 years ($p < 0.05$) (table 1).

In addition table 2 shows a significant relationship between high *cyclin D1* SI and large, poorly-differentiated tumors, carcinomas with positive lymph node metastasis and deeply-invasive carcinomas ($p < 0.05$, $p < 0.001$, $p < 0.05$ and $p < 0.05$ respectively). Whereas *cyclin D1* gene amplification was significantly associated with an advanced disease stage since amplification was detected in

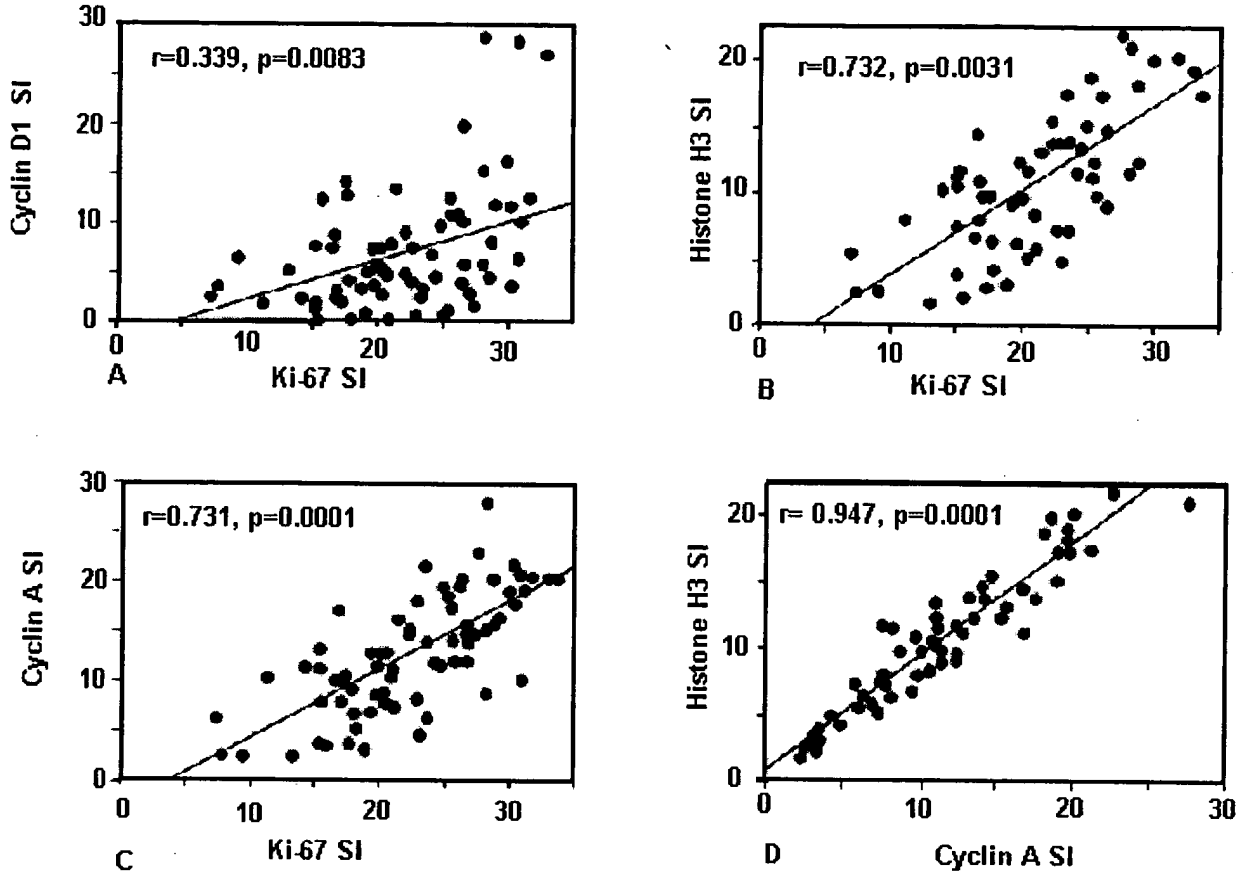


Figure 4
Correlation between the staining intensity of (a) *Ki-67 vs. cyclin D1*, (b) *Ki-67 vs. histone H3*, (c) *Ki-67 vs. cyclin A* and (d) *cyclin A vs. histone H3* mRNA expression.

10/15 (66.7%) of stage IV tumors compared to 12/45 (26.7%) of stage I-III tumors ($p = 0.002$). Similarly, DNA amplification was detected in 60.5% (26/43) of the carcinomas with extensive local invasion (beyond sm) but only in 23.5% (4/17) of the carcinomas with limited invasion (m, sm) ($p = 0.001$). A significant correlation was also present between *cyclin D1* gene amplification and the presence of lymph node metastasis ($p = 0.008$) as well as between the SI of *histone H3*, the size of the tumor and the patient's age ($p < 0.05$, $p < 0.001$ respectively). The SI was higher in tumors >5 cm in diameter and in patients <50 years.

Survival analysis

The mean follow-up period for all patients was 30 months (range: 1-66 months). Eighteen of 60 patients had already died by the time the study was completed. We defined the cutoff level for overexpression of each cell cycle marker at the point that showed the maximum difference of survival rate between the 2 groups separated by that point. Cox regression analysis revealed that *cyclin A* overexpression (our definition: SI ≥ 10.5), *cyclin D1* overexpression (our definition: SI ≥ 6.1), poorly differentiated histology, lymph node metastasis, TNM stage, tumor size and depth of invasion were all significant prognostic variables for survival (Table 3). The Kaplan-Meier survival curves for the subgroups of patients who are subdivided according to each marker's status are shown in Figure 5. Patient with tumors that showed *Ki-67* overexpression (our definition: SI ≥ 11.5) and *histone H3* overexpression (our definition: SI ≥ 8.2) tended to have poor prognosis but this did not reach a statistically significant level, however the overall survival was significantly lower in patient with *cyclin A* and *cyclin D1* overexpression. Cox multivari-

Table 2: The relation between *cyclin D1* overexpression vs *cyclin D1* amplification and clinicopathological prognostic markers.

Variables	*No. of cases*	*Cyclin D1 overexpression*	*Cyclin D1 Amplification*
Tumor size (cm)			
<5.0	33	5.3 ± 3.8*	13/33
≥5.0	27	22.8 ± 7.2 p ***<0.05***	9/27 p *<0.236*
Histology			
GI	15	6.6 ± 4.0	7/15
GII	21	8.9 ± 3.6	8/21
GIII	24	22.0 ± 8.1 p ***<0.001***	7/24 p ***<0.075***
Lymph node			
Negative	33	5.4 ± 5.3*	6/33 (18.2%)
Positive	27	20.6 ± 6.9 p ***<0.05***	16/27 (59.3%) p ***<0.008***
Depth of invasion			
m, sm	17	3.1 ± 3.1*	4/17 (23.5%)
beyond sm	43	12.4 ± 6.5 p ***<0.05***	26/43 (60.5%) p ***<0.001***
Stage			
early	45	5.5 ± 10.1	12/45 (26.7%)
late	15	11.3 ± 9.6 P = *0.175*	10/15 (66.7%) p ***<0.002***

Table 3: Uunivariate analysis of the relationship between survival and the tested markers

PredictiveVariables	*Median Survival*	*HR*	*CI*	*P*
Ki-67				
<11.5	36			
≥11.5	32	1.826	0.636 – 5.243	0.26
Cyclin D1				
<6.1	35			
≥6.1	18	7.246	1.007 – 45.150	0.03*
Histone H3				
<8.2	35			
≥8.2	29	4.639	0.854 – 25.196	0.07
Cyclin A				
<10.5	35			
≥10.5	15	7.820	1.017 – 60.122	0.02*
Histological grade				
Low	38			
High	10	7.331	2.696 – 19.940	0.0001*
Lymph node				
Negative	38			
Positive	15	6.826	1.973 – 23.621	0.002*
Stage				
I, II, III	38			
IV	12	6.378	1.842 – 22.083	0.001*
Tumor size (cm)				
<5.0	35			
≥5.0	13	4.835	1.386 – 16.868	0.01*
Depth of invasion				
T1, T2	36			
T3, T4	20	7.759	1.024 – 58.789	0.04*
Age (years)				
<50	38			
≥50	28	2.802	0.988 – 7.943	0.0526
Sex				
Male	38			
Female	36	0.696	00.274 – 1.766	0.4449

* p. value < 0.05 (significant)
HR: Hazard Ratio
CI: 95% confidence Interval

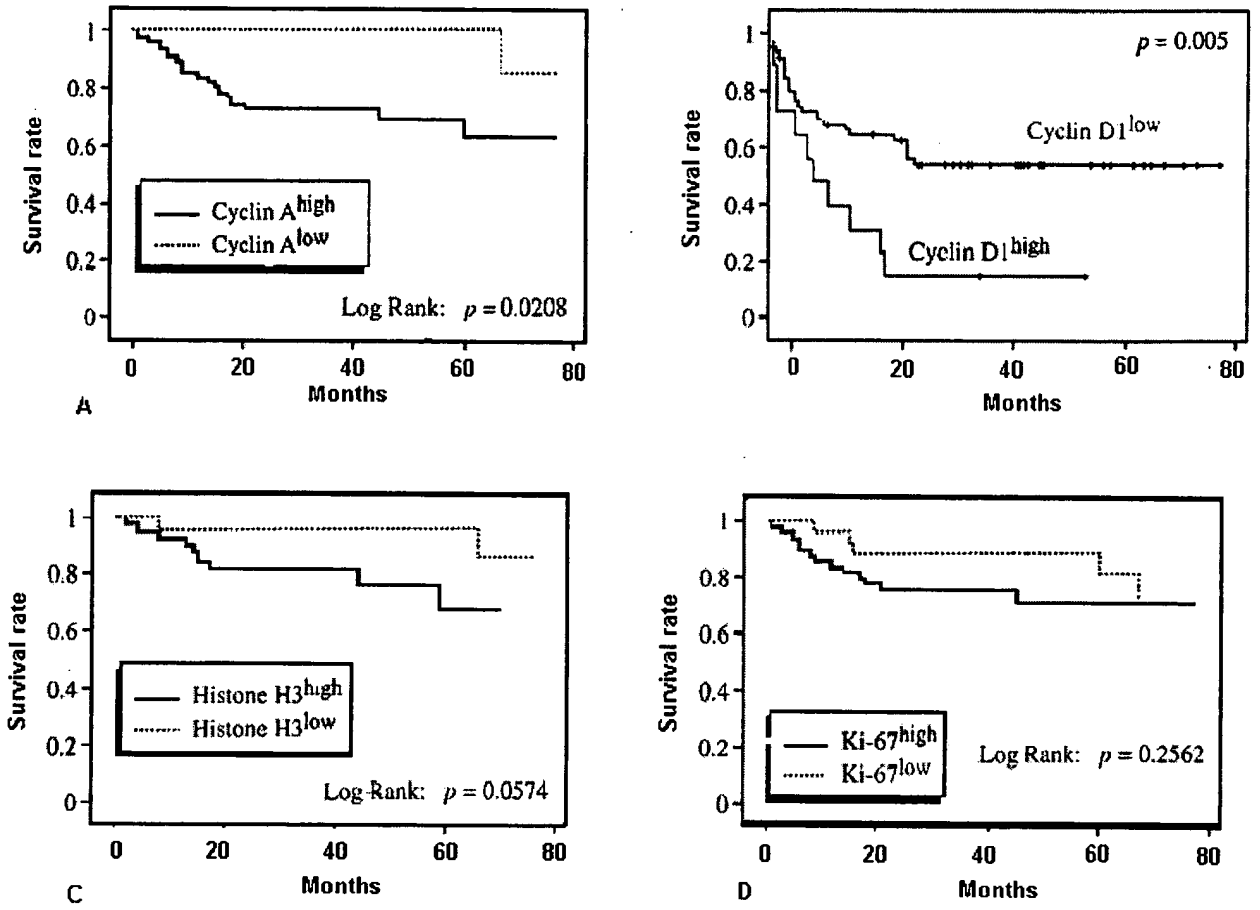


Figure 5
Kaplan-Meier survival curves for colorectal carcinoma. Overall survival is significantly lower in patients with (a) *cyclin A* and (b) *cyclin D1* overexpression. Patients with high SI for *histone H3* mRNA have poorer prognosis but this was not statistically significant (c). No significant difference was present between patients with high *Ki-67* SI and those with low *Ki-67* SI (d).

ate regression analysis revealed that lymph node metastasis, *cyclin A* and *cyclin D1* overexpression were independent negative prognostic factors after adjustment for the depth of tumor invasion, age and sex of the patient (Table 4).

Discussion

The proliferative activity of CRC cells has been investigated in several studies either by immunohistochemical determination of cell proliferation index using antibodies to some types of *cyclins* or by flowcytometric determination of the SPF of the cell cycle [8]. Although Leach et al. [17] did not find *cyclin D1* gene amplification in a panel of 47 CRC cell lines; its protein was overexpressed in about 30% of CRC cases that were included in the studies of Bartakova et al. [6] and Arber et al. [18]. In the former study [6]*cyclin D1* was aberrantly accumulated in a significant subset of human CRC cases and the cell lines derived from these cases were dependent on *cyclin* in their cell cycle progression. In the second study [18], overexpression of *cyclin D1* was detected in 30% of adenomatous polyps indicating that overexpression is a relatively early event in colon carcinogenesis which is possibly responsible for the pathological changes in the mucosa preceding neoplastic transformation. More recently, Holland et al. [19], Pasz-Walczak et al. [20] and Utsunomiya et al. [21] reported up-regulation of *cyclin D1* in 58.7%, 100% and 43% of their studied cases respectively.

Table 4: Multivariate analysis ofthe relationship between survival and thetested markers

PredictiveVariables	HR	CI	P
Cyclin D1	10.864	1.055 – 86.250	0.03*
(baseline < 6.1)	-	-	-
Cyclin A	13.886	1.012 – 190.579	0.0490*
(baseline < 10.5)	-	-	-
Positive Lymph node metastasis	3.921	1.057 – 14.472	0.0410*
Stage IV	3.411	1.048 – 12.083	0.03*
Depth of invasion			
T3, T4	5.408	0.449 – 65.080	0.1836
Age (years)			
≥50	1.996	0.678 – 5.878	0.2310
Sex	0.910	0.315 – 2.358	0.8453

p. value < 0.05 (significant)
HR: Hazard Ratio
CI: 95% confidence Interval

In the present study, up-regulation of *cyclin D1* was detected in 68.3% of the cases. The SI was significantly higher in carcinomas than in normal colorectal mucosa and in poorly-differentiated adenocarcinomas it was approximately twice that of other histological types. Amplification and/or overexpression of *cyclin D1* significantly correlated with deeply invasive tumors and positive lymph node metastasis. Our results in this regards are consistent with previous studies [8,22]. In 2001, Holland et al. [19]. demonstrated that deregulation of *cyclin D1* and $p21^{waf}$ proteins are important in colorectal tumorigenesis and have implications for patient prognosis. Similarly McKay et al. [23] found that *cyclin D1* was the only protein in their panel (*cyclin D1, p53, p16, Rb-1, PCNA and p27*) that correlated with improved outcome in CRC patients. However, few studies failed to detect any correlation between *cyclin D1* overexpression and the clinicopathological factors in CRC [6,18]. This controversy in results could partially be explained by the difference in the sampling of studied cases. The present study included 24 cases of poorly differentiated adenocarcinoma, which is not common in other studies of CRC in western countries. This was possible because the majority of CRC cases diagnosed in Egypt are of high histological grade [3]. The correlation between *cyclin D1* overexpression and the high histological grade was also reported in other tumor types including non-small cell lung carcinomas [24] and squamous cell carcinomas of the larynx [16]. Another possible explanation for the observed controversy in the results of different studies is the detection method used.

In the present work, overexpression of *cyclin D1* was more common than gene amplification of the *PRAD-1/cyclin D1* gene with a 63.6% concordance. This was similarly reported by Bartakova et al. [6] who mentioned that there is a subset of CRC cases in which *cyclin D1* is overexpressed without *PRAD-1/cyclin D1* gene amplification. Consistent with this hypothesis are reports of elevated *cyclin D1* mRNA levels and immunohistochemically detectable accumulation of the protein in over one third of breast cancer cases at a frequency significantly higher than that deduced from DNA amplification studies [9,25]. These data imply that mechanisms other than gene amplification can also lead to deregulation and accumulation of *cyclin D1* in solid tumors.

So far, several studies were done to reveal the prognostic significance of *cyclin D1* overexpression in various carcinomas, including CRC [22]. However, these studies yielded conflicting results which could be attributed to organ heterogeneity. In our study, patients with tumors that exhibited *cyclin D1* overexpression tended to have poor prognosis.

It was reported that, patients with *cyclin A* positive carcinomas had significantly shorter median survival times. Handa et al. [8] were able to detect *cyclin A* overexpression in 77% of their CRC cases. They also demonstrated that, *cylcin A* could be used as a prognostic factor of CRC. More recently, Habermann et al. [26] studied cases of ulcerative colitis with and without an associated adenocarcinoma for the presence of *cyclin A* overexpression. They found that, *cyclin A* overexpression was higher in cases of ulcerative colitis with adenocarcinomas than in those without adenocarcinomas. Consequently, they concluded that, *cyclin A* could be used for monitoring ulcerative colitis patients and for the early detection of an emerging carcinoma in this high risk group of patients.

In our study, *cyclin A* was detected in 80% of the patients and Cox regression analysis showed that it could be used as a prognostic marker in CRC in addition to *cyclin D1*.

It would have been useful if we assessed the expression level of *cyclin A* by another technique (DNA amplification). This would have added more information regarding the gene status on one hand and confirmed the results of IHC on the other hand. Unfortunately, this was not possible because in most of the cases included in the present work, the extracted DNA was not sufficient to study *cyclin amplification* after the assessment of *cyclin D1*.

In 1996, Nagao et al. [11] reported that *histone H3* labeling index significantly correlated with ki-67 immunostaining and was high in poorly differentiated human hepatocellular carcinoma. This was similarly reported in the present work since we found a significant correlation between the SI of *histone H3* and *Ki-67*. However, no

statistically significant correlation was found between *histone H3* SI and any of the studied clinicopathological factors.

Although *Ki-67* immunostaining reflects the proliferative activity of CRC, it has not been recognized as a significant prognostic factor in this type of tumors [27,28]. However, Suzuki at al. [29] found a significant correlation between *Ki-67* labeling index and local invasion of CRC. In the present study there was a significant relationship between the SI of *Ki-67*, tumor size and grade. However, Kaplan-Meier survival curves showed no significant difference in survival rates between patients with- and without overexpression of *Ki-67*.

Conclusions

Our results demonstrate that *cyclin D1*, *cyclin A*, *histone H3* and *Ki-67* are overexpressed in a subset of CRC, however only *cyclin D1* and *cyclin A* overexpression correlates with poor differentiation and tumor progression. This indicates the superiority of *cyclin A* and *cyclin D1* as indicators of poor prognosis compared to *Ki-67* and *histone H3* mRNA in CRC. *Cyclin A and D1* could therefore be considered significant, independent prognostic factors in CRC patients. These findings are especially important in stage II patients since 25–30% of those patients have poor prognosis in spite of being node-negative. However, the standard clinicopathologic prognostic factors can not identify this subset accurately and therefore; there is a great demand for more accurate, individually-based, biological prognostic parameters that help in detecting this high risk group of patients who can benefit from an adjuvant therapy. If the findings of the present study are confirmed in a larger study, evaluation of *cyclin A and D1* may be applicable to clinical management of CRC, allowing the identification of patients with poor prognosis.

Competing interests

The author(s) declare that they have no competing interests.

List of abbreviations

CRC - Colorectal cancer

OS - overall survival

SI - staining index

SPF - S phase fraction

ISH - in situ hybridization

m - muscularis mucosa

sm - invasion of the sub mucosa

Authors' contributions

BA and ZA-R carried out the molecular genetic studies, designed, coordinated the study and drafted the manuscript. BA and El-HS carried out all the histopathological and immunohistochemical studies. El-SA participated in molecular genetic studies and drafted the manuscript. MM coordinated the study. El-SM carried out all the patient clinical data. All authors read and approved the final manuscript

References

1. Jiang GL, Huang S: **Adenovirus expressing RIZ1 in tumor suppressor gene therapy of microsatellite unstable colorectal cancers.** *Cancer Res* 2001, **61**:1796-1798.
2. Soliman AS, Bondy ML, Levin B, Hamza MR, Ismail K, Ismail S, Hammam HM, El-Hattab O, Kamal SM, Soliman AG, Dorgham LA, McPherson RS, Beasley RP: **Colorectal cancer in Egyptian patients under 40 years of age.** *Int J Cancer* 1997, **71**:26-30.
3. Soliman AS, Bondy ML, Guan Y, El-Badawy S, Mokhtar N, Bayomi S, Raouf AA, Ismail S, McPherson RS, Abdel-Hakim TF, Beasley PR, Levin B, Wei Q: **Reduced expression of mismatch repair genes in colorectal cancer patients in Egypt.** *Int J Oncol* 1998, **12**:1315-1319.
4. Cordon-Cardo C: **Mutations of cell cycle regulators. Biological and clinical implications for human neoplasia.** *Am J pathol* 1995, **147**:545-560.
5. Hunter T, Pines J: **Cyclins and cancer. II. Cyclin D and CDK inhibitors come of age.** *Cell* 1994, **79**:573-528.
6. Bartkova J, Lukas J, Strauss M, Bartek J: **The *PRAD-1/cyclin D1* oncogene product accumulates aberrantly in a subset of CRCs.** *Int J Cancer* 1994, **58**:568-573.
7. Motokura T, Arnold A: **Cyclins and oncogenesis.** *Biochim Biophys Acta* 1993, **1155**:63-78.
8. Handa K, Yamakawa M, Takeda H, Kimura S, Takahashi T: **Expression of the cell cycle markers in colorectal carcinoma: Superiority of cyclin A as an indicator of poor prognosis.** *Int J cancer* 1999, **84**:225-233.
9. Gillett C, Fantl V, Smith R, Fisher C, Bartek J, Dickson C, Barnes D, Peters G: **Amplification and overexpression of cyclin D1 in breast cancer detected by immunohistochemical staining.** *Cancer Res* 1994, **54**:1812-1817.
10. Gown AM, Jiang JJ, Matles H, Skelly M, Goodpaster T, Cass L, Reshatof M, Spaulding D, Coltrera DM: **Validation of the S-phase specificity of histone (H3) in situ hybridization in normal and malignant cells.** *J Histochem Cytochem* 1996, **44**:221-226.
11. Nagao T, Ishida Y, Kondo Y: **Determination of S-phase cells by in situ hybridization for histone H3 mRNA in hepatocellular carcinoma: correlation with histological grade and other cell proliferative markers.** *Mod Pathol* 1996, **9**:99-104.
12. Chou MY, Chang AL, McBride J, Donoff B, Gallagher GT, Wong DT: **A rapid method to determine proliferation patterns of normal and malignant tissues by H3 mRNA in situ hybridization.** *Am J Pathol* 1990, **136**:729-733.
13. Sobin LH, Wittekind C: **TNM classification of malignant tumors.** 5th edition. John Wiley, New York; 1997.
14. King RJ, Coffer AI, Gilbert J, Lewis K, Nash R, Millis R, Raju S, Taylor RW: **Histochemical studies with a monoclonal antibody raised against a partially purified soluble estradiol receptor preparation from human myometrium.** *Cancer Res* 1985, **45**:5728-5733.
15. Slebos RJ, Boerrigter L, Evers SG, Wisman P, Mooi WJ, Rodenhuis S: **A rapid and simple procedure for the routine detection of ras point mutations in formalin-fixed, paraffin-embedded tissues.** *Diag Moln Path* 1992, **1**:136-141.
16. Jares P, Fernandez P, Campo E, Nadal A, Bosch F, Aiza G, Nayach I, Traserra J, Cardesa A: ***PRAD-1/cyclin D1* gene amplification correlates with messenger RNA overexpression and tumor progression in human laryngeal carcinomas.** *Cancer Res* 1994, **54**:4813-4817.
17. Leach FS, Elledge SJ, Sherr CJ, Willson JK, Markowitz S, Kinzler KW, Vogelstein B: **Amplification of cyclin genes in colorectal carcinomas.** *Cancer Res* 1993, **53**:1986-1989.

18. Arber N, Hibshoosh H, Moss SF, Sutter T, Zhang Y, Begg M, Wang S, Weinstein IB, Holt PR: **Increased expression of cyclin D1 is an early event in multistage colorectal carcinogenesis.** *Gastroenterology* 1996, **110**:669-674.
19. Holland TA, Elder J, McCloud JM, Hall C, Deakin M, Fryer AA, Elder JB, Hoban PR: **Subcellular localization of cyclin D1 protein in colorectal tumors is associated with p21 (WAF1/CIP1) expression and correlates with patient survival.** *Int J Cancer* 2001, **95(5)**:302-306.
20. Pasz-Walczak G, Kordek R, faflik M: **P21(WAF1) expression in colorectal cancer: correlation with p53 and cyclin D1 expression, clinicopathological parameters and prognosis.** *Pathol Res Pract* 2001, **197(10)**:683-689.
21. Utsunomiya T, Doki Y, Takemoto H, Shiozaki H, Yano M, Sekimoto M, Tamura S, Yasuda T, Fujiwara Y, Monden M: **Correlation of beta-catenin and cyclin D1 expression in colon cancers.** *Oncology* 2001, **61(3)**:226-233.
22. Maeda K, Chung YS, Kang SM, Ogawa M, Onoda N, Nakata B, Nishiguchi Y, Ikehara T, Okuno M, Sowa M: **Overexpression of cyclin D1 and p53 is associated with disease recurrence in colorectal adenocarcinoma.** *Int JCancer* 1997, **74**:310-315.
23. McKay JA, Douglas JJ, Ross VG, Curran S, Loane JF, Ahmed FY, Cassidy J, McLeod HL, Murray GI: **Analysis of key cell cycle checkpoint proteins in colorectal tumors.** *J Pathol* 2002, **196**:386-393.
24. Mate JL, Ariza A, Aracil C, Lopez D, Isamat M, Perez-Piteira J, Navas-Palacios JJ: **Cyclin D1 overexpression in non-small cell lung carcinoma: correlation with Ki-67 labeling index and poor cytoplasmic differentiation.** *J Pathol* 1996, **180**:395-399.
25. Buckley MF, Sweeney KJ, Hamilton JA, Sini RL, Manning DL, Nicholson RI, DeFazio A, Watts CK, Musgrove EA, Sutherland RL: **Expression and amplification of cyclin genes in human breast cancer.** *Oncogene* 1993, **8**:2127-2133.
26. Habermann J, Lenander C, Roblick UJ, Kruger S, Ludwig D, Alaiya A, Freitag S, Dumbgen L, Bruch HP, Stange E, Salo S, Tryggvason K, Auer G, Schimmelpenning H: **Ulcerative colitis and colorectal carcinoma: DNA profile, laminin-5 gamma 2 chain and cyclin A expression as early markers for risk assessment.** *Scand J Gastroenterol* 2001, **36**:751-758.
27. Kubota Y, Petras RE, Easley KA, Bauer TW, Tubbe RR, Fazio VW: **Ki-67-determined growth fraction versus standard staging and grading parameters in colorectal carcinoma. A multivariate analysis.** *Cancer* 1992, **70**:2602-2609.
28. Shain AA, Ro JY, Brown RW, Ordonez NG, Cleary KR, El-Naggar AK, Wilson P, Ayala AG: **Assessment of Ki-67-derived tumor proliferative activity in colorectal adenocarcinomas.** *Mod Pathol* 1994, **7**:17-22.
29. Suzuki H, Matsumoto K, Terabe M: **Ki-67 antibody labeling index in colorectal carcinoma.** *J clin Gastroenterol* 1992, **15**:317-320.

Pre-publication history

The pre-publication history for this paper can be accessed here:

http://www.biomedcentral.com/1471-230X/4/22/prepub

British Journal of Cancer (2004) 90, 1612–1619

www.bjcancer.com

Correlation of amplification and overexpression of the c-*myc* oncogene in high-grade breast cancer: FISH, *in situ* hybridisation and immunohistochemical analyses

J Blancato*[,1,2], B Singh[2], A Liu[3], DJ Liao[4] and RB Dickson[2,5]

[1]Institute for Molecular and Human Genetics, 3970 Reservoir Road, NW, Washington DC 20007, USA; [2]Lombardi Comprehensive Cancer Center, 3970 Reservoir Road, NW, Washington DC 20007, USA; [3]Biometry and Mathematical Statistics Branch, National Institute of Child Health and Human Development, 9000 Rockville Pike, Bethesda, MD 20892-7510, USA; [4]Department of Radiation Oncology, Karmanos Cancer Institute, Wayne State University, Detroit, MI, USA; [5]Department of Oncology, Georgetown University Medical Center, 3970 Reservoir Road, NW, Washington DC 20007, USA

In this study, we analysed gene amplification, RNA expression and protein expression of the c-*myc* gene on archival tissue specimens of high-grade human breast cancer, using fluorescent *in situ* hybridisation (FISH), nonradioactive *in situ* hybridisation and immunohistochemistry. The specific question that we addressed was whether expression of c-*Myc* mRNA and protein were correlated with its gene copy amplification, as determined by FISH. Although c-*Myc* is one of the most commonly amplified oncogenes in human breast cancer, few studies have utilised *in situ* approaches to directly analyse the gene copy amplification, RNA transcription and protein expression on human breast tumour tissue sections. We now report that by using the sensitive FISH technique, a high proportion (70%) of high-grade breast carcinoma were amplified for the c-*myc* gene, irrespective of status of the oestrogen receptor. However, the level of amplification was low, ranging between one and four copies of gene gains, and the majority (84%) of the cases with this gene amplification gained only one to two copies. Approximately 92% of the cases were positive for c-*myc* RNA transcription, and essentially all demonstrated c-myc protein expression. In fact, a wide range of expression levels were detected. Statistically significant correlations were identified among the gene amplification indices, the RNA expression scores and protein expression scores. c-*myc* gene amplification, as detected by FISH, was significantly associated with expression of its mRNA, as measured by the intensity of *in situ* hybridisation in invasive cells ($P = 0.0067$), and by the percentage of invasive cells positive for mRNA expression ($P = 0.0006$). c-*myc* gene amplification was also correlated with the percentage of tumour cells which expressed high levels of its protein, as detected by immunohistochemistry in invasive cells ($P = 0.0016$). Thus, although multiple mechanisms are known to regulate normal and aberrent expression of c-*myc*, in this study, where *in situ* methodologies were used to evaluate high-grade human breast cancers, gene amplification of c-*myc* appears to play a key role in regulating expression of its mRNA and protein.
British Journal of Cancer (2004) **90,** 1612–1619. doi:10.1038/sj.bjc.6601703 www.bjcancer.com
Published online 30 March 2004

Keywords: c-*myc*; breast cancer; gene amplification; gene expression

Genetics and Genomics

The c-*myc* oncogene has been shown to be amplified and/or overexpressed in many types of human cancer (Marcu *et al*, 1992; Nass and Dickson, 1997; Nesbit *et al*, 1999; Liao and Dickson, 2000). Numerous experiments *in vivo* have also causally linked aberrant expression of this gene to the development and progression of cancer in different body sites (Marcu *et al*, 1992; Nass and Dickson, 1997; Nesbit *et al*, 1999; Liao and Dickson, 2000). However, several critical issues regarding the significance of c-*myc* in human cancer still remain obscure. First, even for a given type of malignancy, the frequencies of the alterations of c-*myc* at the cytogenetic and expression levels vary greatly from one report to another (Liao and Dickson, 2000). For instance, the frequencies of its amplification, mRNA and protein overexpression in breast cancer vary between 1–94, 22–95 and roughly 50–100%, respectively, among different reports (Liao and Dickson, 2000). Thus, it is still unclear to what extent this gene is altered at the cytogenetic level and at different expression levels in breast carcinoma.

One controversial issue pertains to the prognostic value of c-*myc* gene alterations in cancer. The central role of c-Myc protein in accelerating cell proliferation, documented by many early studies, has led to a general concept for many types of cancer that amplification or overexpression of this gene may be associated with a more aggressive tumour and a poorer patient survival (Berns *et al*, 1992; Marcu *et al*, 1992; Sato *et al*, 1995; Nass and Dickson, 1997; Nesbit *et al*, 1999; Visca *et al*, 1999; Liao and Dickson, 2000). However, many reports have shown an opposite correlation (Sikora *et al*, 1985, 1987; Watson *et al*, 1986; Polacarz *et al*, 1989; Voravud *et al*, 1989; Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Diebold *et al*, 1996; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999), while other studies do not support either of these conclusions. For instance, gene amplification or overexpression of c-Myc protein has also been shown to associate with a better tumour differentiation or a better

*Correspondence: Dr J Blancato; E-mail: blancatj@georgetown.edu
Received 7 July 2003; revised 17 January 2004; accepted 17 January 2004; published online 30 March 2004

patient survival for cancer of the testis, ovary, bile ducts, colon and breast (Sikora *et al*, 1985, 1987; Watson *et al*, 1986; Polacarz *et al*, 1989; Voravud *et al*, 1989; Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Diebold *et al*, 1996; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999). This controversy does not appear to be related completely to the cancer type, since both positive (Berns *et al*, 1992; Visca *et al*, 1999) and negative (Williams *et al*, 1990; Melhem *et al*, 1992; Pietilainen *et al*, 1995; Smith and Goh, 1996; Augenlich *et al*, 1997; Bieche *et al*, 1999) correlations have been reported for colon cancer and breast cancer. More interestingly, c-Myc overexpression has been shown to predict a poorer prognosis for cutaneous melanoma, but a favourable outcome for uveal melanoma (Grover *et al*, 1997; Chana *et al*, 1998a, b, 1999; Grover *et al*, 1999). These data indicate different roles of c-Myc, even in the same type of tumour, perhaps depending upon different tissue microenvironments.

Another controversial issue concerns the nuclear–cytoplasmic localisation of c-Myc. Studies of neoplasms of the colon, testis, ovary and liver have shown that predominantly nuclear localisation of c-Myc tends to occur in benign lesions, while cytoplasmic localisation tends to occur in more malignant tumours (Sikora *et al*, 1985; Sundaresan *et al*, 1987; Melhem *et al*, 1992; Sasano *et al*, 1992; Yuen *et al*, 2001). Whether these patterns of subcellular localisation of c-Myc tend to reflect the malignant status of breast cancer remains an enigma.

A recent study of the impact of DNA amplification on gene expression patterns in breast cancer used mRNA and DNA from 14 breast cancer cell lines. Analysis was conducted with a 13 000 cDNA clone array for gene expression measurement and a Comparative Genomic Hybridisation (CGH) microarray for gene copy number measurements. This study also included known breast cancer genes, such as *c-myc*, *HER/2-neu* and *aib1* (Hyman *et al*, 2002). Interestingly, 44% of the most highly amplified genes were also overexpressed at the mRNA level. Consistent with this pattern, *c-Myc* gene copy number and its expression levels showed a statistically significant ($\alpha = 0.020$) correlation in this microarray study of breast cancer cell lines. Another study, by Pollack and colleagues, used microarray analysis and BAC array CGH of RNA and DNA (respectively) extracted from intermediate grade human breast tissues, and tested for amplification and expression of c-Myc (among other genes). This study demonstrated that two out of 37 specimens were both amplified and overexpressed, while others were either amplified or overexpressed, but not both. The authors of this study suggested that contaminating stromal tissue may compress the fluorescence ratios leading to underestimates of gene amplification and overexpression (Pollack *et al*, 2002).

To more clearly address the importance of gene amplification and expression of c-Myc in human breast cancer, we used *in situ* methodologies, which can clearly distinguish stromal and carcinoma components. We studied the amplification and over expression of the *c-myc* gene with fluorescent *in situ* hybridisation (FISH), non-radioactive *in situ* hybridisation (ISH) and immunohistochemical (IHC) approaches on paraffin-embedded biopsy sections of untreated, high-grade breast cancer. It was observed that 70, 92 and 70% of the cancer cases exhibited *c-myc* gene amplification, its mRNA overexpression and its protein over expression, respectively. In most of the cases (84%) that showed gene amplification, the *c-myc* gene gained only one to two copies, which is consistent with *c-myc* FISH data from other studies. Unlike some oncogenes, such as *N-myc*, which typically demonstrates gene amplification copy numbers of greater then 10 in neuroblastoma, and *HER-2/neu* (Sartelet *et al*, 2002), whose copy numbers range up to 14–40 in breast carcinomas (Isola *et al*, 1999), gene copy numbers of *c-myc* are not as greatly increased. In the study noted earlier, using breast cancer cell line CGH array and cDNA microarray expression analysis, it was demonstrated that the most dramatically increased expression levels were associated with large gene copy number increases, although low-level gains and losses had a significant influence on gene expression dysregulation (Hyman *et al*, 2002). Only one study has been published (Pollack *et al*, 2002) that has begun to determine if these findings are directly relevant to actual human breast tumour tissues, since many of the genetic changes in tissue culture cell lines are more extreme than those displayed in primary tumour material. Furthermore, the relationships among gene amplification, mRNA expression and c-Myc protein expression were not explored in prior human breast cancer cell line and tumour tissue studies (Hyman *et al*, 2002; Pollack *et al*, 2002).

In our human breast tumour tissue study, a high correlation was found between *c-myc* FISH and ISH, for both percentage of staining ($P<0.0067$) and intensity positive cells ($P<0.0006$). In addition, *c-myc* gene copy amplification by FISH was correlated with c-Myc protein expression positive cells by IHC ($P<0.0016$). These results support the idea that c-Myc overexpression of both mRNA and protein is related to the copy number of the *c-myc* DNA amplification. We show in this study that amplification and overexpression of c-Myc occur with high frequency in high-grade human breast cancer tissues.

MATERIALS AND METHODS

Materials

Formalin-fixed, paraffin-embedded tissue blocks of breast carcinoma and normal breast tissue were obtained from the Histopathology and Tissue Shared Resource at the Lombardi Comprehensive Cancer Center (LCCC), at Georgetown University Medical Center. The criteria for tumour selection were the following: negative progesterone receptor status, metastases to auxiliary lymph nodes and high grade (Elston Score >7). The oestrogen receptor status of the tumours was known from archived pathology reports. The parameters were chosen from our prior meta-analysis (Deming *et al*, 2000), as indications of a high likelihood of *c-myc* gene amplification. Normal breast tissue specimens were from reduction mammoplasty. Serial sections (5 μm) for FISH, ISH and IHC were prepared by the LCCC Histopathology and Tissue Shared Resource.

FISH

A dual-label FISH technique was used (Jenkins *et al*, 1997). Slides were baked overnight at 60°C to assure adherence of the sample. Tissue sections were deparaffinised with two successive, 10 min xylene washes, and then dehydrated in a graded ethanol series of 70, 80 and 95% at room temperature. Samples were then digested with 4% pepsin (Sigma, St Louis, MO, USA) at 45°C for 10 min. DNA probes used were an alpha satellite probe to chromosome 8, labelled with biotin, and a *c-myc* probe, labelled with digoxigenin (Ventana, Tucson, AZ, USA). Codenaturation was performed at 90° for 10 min on a hot plate. Hybridisation was at 37°C for 12–16 h. Detection of signals was accomplished with an antiavidin antibody labelled with Texas Red, and an antidigoxigenin antibody conjugated to fluorescein (Ventana, Tucson, AZ, USA). Slides were postwashed in 2 × SSC at 72°C for 5 min and counterstained with DAPI to visualise cell nuclei. Results were viewed and quantified with a Zeiss Axiophot fluorescence microscope, equipped with appropriate filters and an Applied Imaging Cytovision system (Pittsburgh, PA, USA). In this approach, the *c-myc* unique sequence probe was visualised as a green signal and the control probe for the chromosome 8 centromere was red, thus easily being distinguished when scored.

One serial section from each tumour sample was stained with haematoxylin and eosin and first reviewed by a pathologist (BS), to help identify the tumour area of the section. This procedure ensured that the tumour cells, but not the normal cells, were

counted. Nuclei of up to 50 tumour cells were scored from each FISH-stained section, independently by two investigators. Hybridisation signals were averaged, and the amplification index was presented as the number of c-*myc* signals divided by the number of chromosome 8 centromere signals. A 1.8-fold increase was used as the criterion to judge the presence of c-*myc* gene amplification.

In situ hybridisation

In situ hybridisation (ISH) was carried out with a nonradioactive method, described previously (Liao *et al*, 2000a, b). One serial section from each specimen was hybridised overnight at 60°C with riboprobes, that were *in vitro* transcribed from the antisense or sense strand of an approximately 300 bp cDNA of human c-*myc* (ATCC, Manassas, VA, USA), labelled with digoxigenin-conjugated UTP. The sections were then incubated with an antibody against digoxigenin, followed by incubation with a second antibody conjugated to alkaline phosphatase. The signal was visualised by colour development with 5 bromo-4-chloro-3-indolyl phosphate and nitroblue tetrazolium. All reagents were purchased from Boehringer Mannheim, Indianapolis, IA. To control the signal specificity, two serial sections were mounted on the same slide for hybridisation with the antisense and sense probes, respectively. ISH was given an intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. Intensity scores were assigned 0, 1, 2 and 3, and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%.

Immunohistochemistry

Immunohistochemical staining (IHC) was performed using an avidin–biotin complex (ABC) method described previously (Liao *et al*, 1998). One serial section of each specimen was deparaffinised and blocked with 3% peroxide. Antigens were retrieved by heating slides in a microwave oven in 50 mM citrate buffer, pH 6.4, at boiling temperature, for 12 min. After blocking with 6% normal goat serum, the section was incubated with a mouse monoclonal antibody to human c-Myc (9E10, Sigma Chemical Company, St Louis, MO, USA) at 1:100 dilution for 2 h, followed by 1 h incubation with a second antibody conjugated with biotin (Vector Laboratories Inc., Burlingame, CA, USA). The section was then incubated with peroxidase-conjugated avidin (Dako, Corporation, Carpinteria, CA, USA) for 30 min, followed by colour development with diaminobenzidine and peroxide. All procedures were carried out at room temperature. To control the signal specificity, serial sections from 10 tumour samples were also stained using an alternate c-Myc antibody (C19 from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) at 1:60 dilution. This antibody resulted in focally positive staining in the tumour, but the staining intensity was weaker. To control the signal specificity, serial sections were made from five selected positive cases which were subjected to the same staining procedure, with a normal mouse IgG to replace the c-Myc antibody. This control staining did not give rise to a signal, demonstrating the specificity of the c-Myc antibody signal. IHC staining was given an intensity and percentage score based upon the intensity of positive staining and number of cells staining. Intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%. Determinations were made of cellular localisation of c-Myc antibody staining to cytoplasm and/or nucleus in normal and invasive cells within each breast tumour specimen.

Statistical analyses

For each analysis of gene copy amplification (FISH), mRNA expression (ISH) and protein expression (IHC), all cases were first grouped as positive or negative to calculate the percentages of positive cases and negative cases, as described (Zar, 1974). Fisher's exact test was used to compare percentages, and two-sample *t*-test or Wilcoxon rank test was used to compare average scores. Both ISH and IHC were given intensity and percentage scores, based on intensity of positive staining and number of cells staining, respectively. As noted earlier, intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 1- 1–25, 2- 26–50, 3- 51–75 and 4- 76–100%. A score of >2 for either intensity of staining or percentage of cells positive by ISH was assigned as high. For IHC, an intensity score of >1 was assigned as high and a percentage score of >3 was categorised as high. Each amplification index was paired with its corresponding mRNA expression score to calculate the coefficient *r*. The same method was used to estimate the association of the amplification indices with the c-Myc protein expression levels, and the association of the mRNA expression levels with the protein expression levels. A *P*-value of 0.05 or less was used to determine the statistical significance in all analyses. In all, 54 pairs of normal *vs* invasive tissues were analysed using McNemars χ^2 test to determine if there was a difference in cellular localisation of c-Myc antibody signal to nuclear or cytoplasmic compartments.

RESULTS

FISH analysis of gene amplification

Amplification of the c-*myc* gene was measured by a FISH test in 46 cases of breast cancer; Figure 1 demonstrates cells with no amplification (one copy of c-*myc* /one copy of chromosome 8 centromere, and a moderate amplification a 3/1 ratio). Amplification was calculated by the number of c-*myc* signals divided by the number of chromosome 8 alpha satellite signals. A 1.8-fold increase cut-off was used to judge gene amplification. As shown in Table 1, 32 out of 46 (70%) cases were gene amplified for c-*myc*, whereas only 30% (14/46) of the cases showed amplification indices lower than the cut-off value. The amplification indices for most (84%, or 27/32) cases with gene amplification, ranged between 1.8- and three-fold, indicating that the locus gained up to two copies of c-*myc* in the majority of the cases. The percentage of cases with gene gains of three copies or higher was 11% (five out of 46) of total cases analysed, or near 16% (five out of 32) of the cases with gene amplification, including one case (2% of total cases or 3% of the cases with gene amplification) with the highest index of 5 (a gain of four copies).

In all, 28 of the breast carcinomas in this study were ER negative, and 14 were ER positive. The average c-*myc* gene amplification score was 1.896 (s.e. = 0.196) for ER positive and 2.201 (s.e. = 0.157) for ER negative. Although ER-negative tumours had a slightly higher average c-*myc* score, the difference was not statistically significant (two-sided $P = 0.252$ from two-sample *t*-test and 0.251 from Wilcoxon rank test), consistent with the results of our prior meta-analysis of the literature (Deming *et al*, 2000).

In situ hybridisation analysis of c-*myc* mRNA expression

A total of 51 breast cancer samples were studied for c-Myc mRNA expression, with non radioactive *in situ* hybridisation (ISH). ISH results were assigned intensity and percentage scores based upon signal intensity of positive staining and number of cells staining within the sample, respectively. As shown in Table 2, 86% (44 out of 51) tumours were scored as high in intensity, and 92% (47out of 51) had more than 51% positive cells, also considered as highly increased c-Myc expression. mRNA expression was heterogeneous in the breast tumour tissue, and no morphologic subtype was predominant in the high or low categories. One case showed no c-Myc ISH staining. In 79% (38/48) of cases, epithelia in normal mammary glands adjacent to the tumour also showed a high

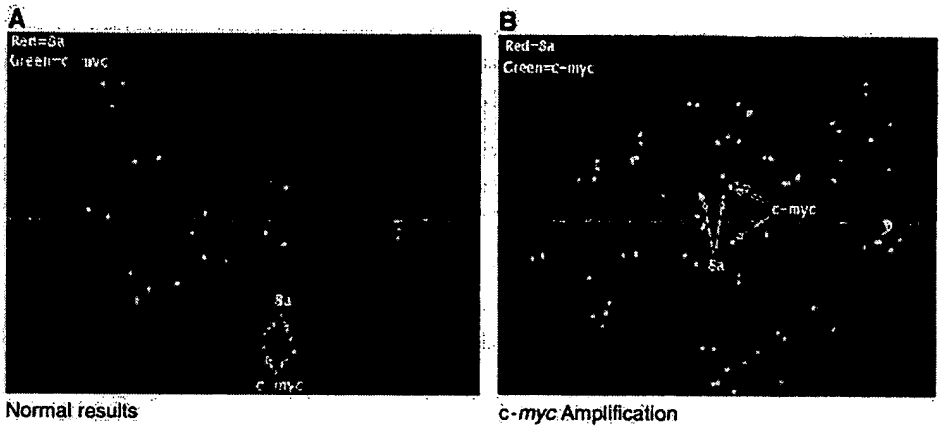


Figure 1 FISH analysis of c-*myc* amplification in tumour cells from breast tumour tissue sections. FISH probe for human c-*myc* unique-sequence is seen as green, while the normal control signal, a centromeric probe signal for chromosome 8 is shown in red. The nuclei of tumour cells were visualised by DAPI counter-staining. (**A**) 1 : 1 copy ratio of c-*myc* to chromosome 8 (c-*myc*/8 centromere), indicating no amplification of c-*myc* in tumour cells. (**B**) 1 : 3 copy ratio of c-*myc* to chromosome 8 (c-*myc*/8 centromere), a moderate amplification of the c-*myc* gene.

Table 1 c-*myc* gene copy amplification analysis by FISH in poor prognosis human breast tumour samples

Amplification index (#c-*myc* signals/# control signals)+	Percentage of samples with FISH ratios in each category	
1.0–1.7	30%	14 out of 46
1.8–1.99	20%	Nine out of 46
2.0–2.9	39%	18 out of 46
>3.0	11%	Five out of 46

Analysis was conducted on 46 individual paraffin-embedded tissue samples with negative progesterone receptor status, positive lymph node involvement and high tumour grade. +Normal control ratio is 1.

intensity of staining. In three cases, no staining was seen in the normal terminal duct lobular units. Figure 2 shows representative fields of high, medium and low *c-myc* mRNA expression levels in invasive ductal carcinoma samples.

Association of FISH and ISH

c-Myc scores were dichotomised as binary variables (high or low), and a score of 2 or higher was categorised as high on ISH. A score higher than median was categorised as high from FISH studies. These dichotomised scores are depicted in Table 3. A Fisher's exact test was performed for comparing binary responses to see if there was any association between FISH and ISH. It was found that the FISH score was significantly associated with percentage of staining in the invasive cells ($P=0.0067$, two-sided McNemar's test) and also with the intensity score on ISH ($P=0.0006$, two-sided).

Immunohistochemical staining of c-Myc proteins

In total, 51 breast carcinomas, which were subjected to FISH analysis, and all of which also had been analysed for c-*myc* mRNA by *in situ* hybridisation, were also analysed for the expression of c-Myc protein, using immunohistochemical staining with the 9E10 antibody. IHC results were assigned an intensity and percentage score based on intensity of positive staining and number of cells staining, respectively. Intensity scores were assigned 0, 1, 2 and 3 and percentage scores were assigned as 0, 1- 0–25, 2- 26–50, 3- 51–75 and 4- 76–100. For IHC, an intensity score of >1 was assigned as high and a percentage score of >3 was categorised as high. Figure 2 shows examples of high, medium and low levels of c-myc antibody staining in invasive ductal carcinoma samples. In 34 cases, normal tissue was seen; 30 of these showed cytoplasmic staining and 22 had nuclear staining in terminal ductal lobular units. In all, 12 cases showed 1+, 14 cases 2+ and four cases 3+ cytoplasmic staining. *In situ* hybridisation revealed positive staining in 46 out of 49 cases with normal tissue. Seven cases showed 1+, 13 cases showed 2+ and 26 cases showed 3+ staining by ISH. Both immunohistochemistry and *in situ* hybridisation showed diffuse positivity in adipocytes.

Table 4 shows the staining pattern for the cohort. In all, 70% (36 out of 51) of cases showed high intensity of staining for c-Myc protein, while 85% (29 out of 34) of cases with detectable staining had more than 76% positive cells, also considered as high expression. To verify the staining specificity, serial sections from 10 tumour specimens that were positive for 9E10 antibody were also stained using the C19 rabbit polyclonal anti-c-Myc antibody. Results revealed a staining pattern similar to 9E10. However, the staining intensity with C19 was weaker than 9E10. The specificity of these two antibodies was verified by Western blots in previous studies (Persons *et al*, 1997; Liao *et al*, 2000b). Figure 2 shows results of c-Myc *in situ* hybridisation and immunohistochemistry studies on samples considered to demonstrate low, moderate and high levels of c-Myc expression. Analysis of c-Myc protein localisation results in the nucleus or cytoplasmic compartments of normal and invasive cells within the tumours revealed that nuclear staining was positive in 41% of normal cells, compared to 22% of invasive cells (statistical significance at $P=0.01$ by McNemar's two-sided χ^2 test). The increase in relative cytoplasmic localisation of c-Myc protein, comparing normal (53.7%), to invasive cells (61.1%) was not significantly different. Thus, the data are consistent with partial exclusion of c-Myc from the nuclei of invasive breast cancer cells.

The FISH score was significantly associated with the percentage positivity of invasive cells, as seen on IHC studies of c-Myc. However, 40% of tumours displayed a low index of c-*myc* gene amplification, but still expressed high levels of c-Myc protein (Table 6), indicating the possibility of other mechanisms of over expression unrelated to gene amplification in at least some tumours. The FISH score was not significantly associated with the intensity of IHC staining in the invasive cells (not shown), in contrast to the IHC percentage positivity score.

Table 2 c-myc mRNA *in situ* hybridisation (ISH) results

Staining intensity	0	1	2	3	Percent positivity	1	2	3	4
Number of tumour samples in each category $N=51$	1	6	25	19	Number of tumour samples in each level category $N=51$	1	3	5	42

In all, 51 human high-grade breast carcinomas were analysed to determine the relationships between c-Myc mRNA expression and c-*myc* gene *in situ* hybridisation results. Data are shown in two ways in the above table. First, overall staining intensity of c-Myc-positive cells was scored as 0, 1, 2, 3 (low to high), and the number of tumour samples at each level of staining indicated on the line below. Next, the percentage of tumour cells staining was scored as 0, 1, 2, 3, 4 (low to high %, as discussed in Materials and Methods). The number of tumours at each level of percent cell positivity for c-Myc is then indicated on the line below.

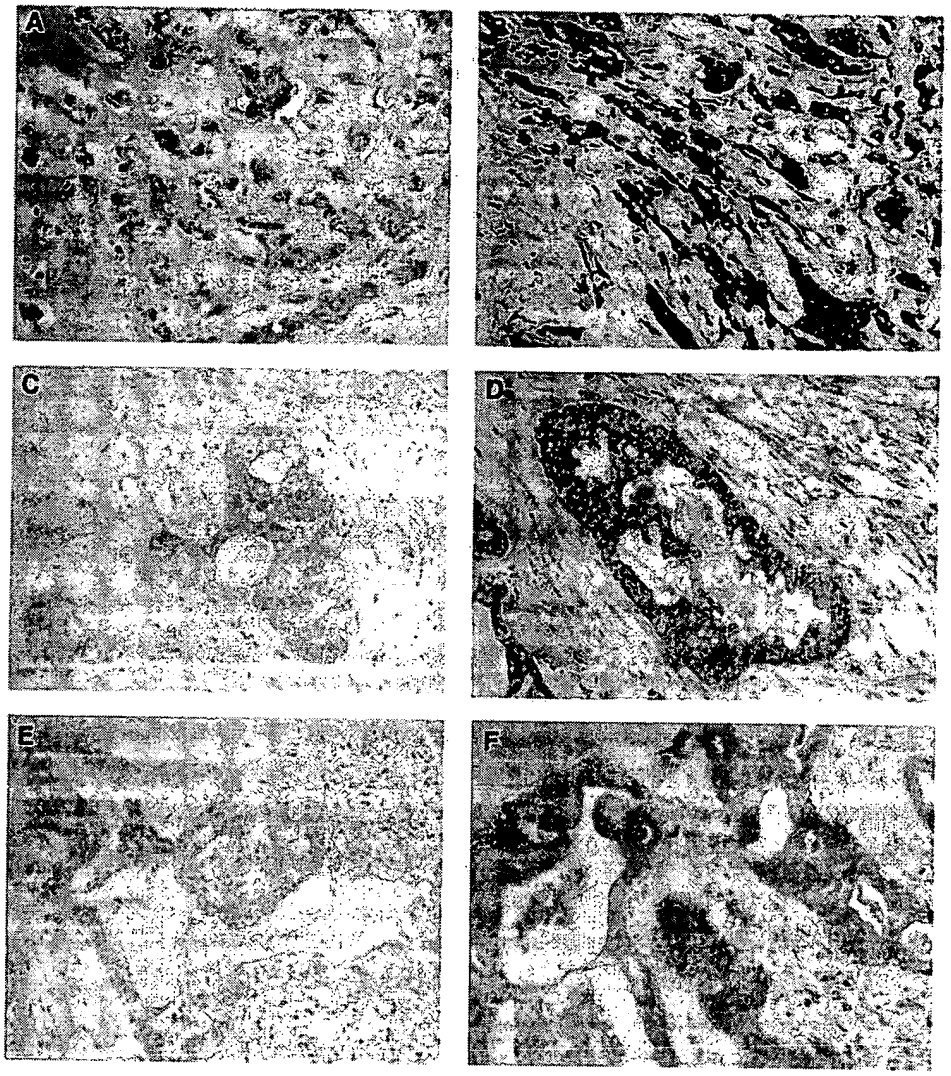


Figure 2 Immunohistochemical staining and *in situ* hybridisation for c-Myc of three sets of invasive ductal carcinoma. (**A**, **C** and **E**) High (3+), intermediate (2+) and low (1+) level of staining by immunohistochemistry for c-Myc. (**B**, **D** and **F**) High (3+), intermediate (2+) and low (1+) level of staining by *in situ* hybridisation.

DISCUSSION

Although there have been many reports on c-*myc* amplification in human breast cancer (Liao and Dickson, 2000), there are only two published studies involving application of the FISH technique to unfixed, frozen sections (Persons *et al*, 1997; Visscher *et al*, 1997), and one prior study using FISH on an archival human tissue microarray (Schraml *et al*, 1999). Another recent study applied FISH to evaluate c-*myc* amplification in ductal carcinoma *in situ* (DCIS) (Aulmann *et al*, 2002). Using the FISH technique on formalin-fixed, paraffin-embedded sections, we now show that 70% of high-grade breast cancer samples bear c-*myc* gene copy amplifications. Interestingly, the above-mentioned study, using FISH and focusing on DCIS, detected amplification of c-*myc* in only 20% of cases, but found a correlation of c-*myc* with increased tumour size and proliferation (Aulmann *et al*, 2002).

The level of amplification of c-*myc* in our study ranged between one and four additional copies of the gene; the majority (84%) of

the cases with the gene amplification gained only one to two copies, also consistent with FISH data reported for c-*myc* copy amplification in human metastatic prostate carcinoma tissues (Jenkins *et al*, 1997). The relationship between the level of c-*myc* gene copy amplification and the level its increased mRNA expression has been examined previously in breast cancer cell lines (Hyman *et al*, 2002). In general, it has been concluded that the two scores coordinate for c-*myc*, as is the case for many breast cancer genes. However, only 44% of the highly amplified genes, in general, showed increased RNA expression, and only 10.5% of the highly overexpressed genes were gene copy-amplified in the cell line study (Hyman *et al*, 2002). Another analysis was conducted to study of relationships between gene amplification and expression of 6095 genes in 37 intermediate grade human breast tumours. This study demonstrated that 62% of the highly amplified genes also showed elevated expression; overall, a two-fold change in DNA copy number was associated with a 1.5-fold change in mRNA levels. Overall, 12% of the variation in gene expression in the breast tumours studied was associated with gene copy number variation (Pollack *et al*, 2002). Further study of additional human breast tumours, at precisely defined grades and stages, will be necessary in order to more fully define the relationships between DNA copy numbers and expression of genes. The studies we report here indicate higher levels of c-Myc gene amplification and expression, than other previous reports in breast cancer. We believe that this is probably the result of our analysis of individual tumour cells in a well-defined set of high-grade breast tumours. Prior c-Myc expression and amplification microarray studies used tumour specimens which contain normal stromal components, potentially underestimating amplification and expression levels of the invasive tumour components (Pollack *et al*, 2002).

Our study reports a percentage of tumours gene amplified for c-*myc* (using FISH in high-grade tumours) that is much higher than the average figure (15.5%) reported in the literature (Isola *et al*, 2002). Most of the prior studies have employed the relatively insensitive Southern blot technique, and were reviewed in a recent meta-analysis (Deming *et al*, 2000). Consistent with this prior literature background, a recent study of 94 lobular and ductal breast cancers assessed amplification of c-*myc* by using a semiquantitative PCR assay and protein expression, with

Table 3 Correlations between c-*myc* gene copy number (FISH) mRNA expression (ISH)

	FISH	
	Low	**High**
(A) ISH (% cells)		
Low	*1*	3
High	*19*	*18*
		$P=0.0067$
(B) ISH (intensity)		
Low	*2*	*5*
High	*18*	*16*
		$P=0.0006$

Serial sections of high-grade human breast carcinomas were scored for c-*myc* gene copy number (FISH, Table 1) and mRNA expression (ISH, Table 2). In (A), a positive correlation ($P=0.0067$) was observed between tumour samples with a high percentage of cells demonstrating mRNA expression and a high c-*myc* gene copy number. A score of 2 or higher was classified as high on ISH, and a score of median or greater was categorised as high on FISH. In (B), a positive correlation ($P=0.0006$) was shown between a high level of intensity for c-Myc RNA expression a high and c-*myc* gene copy number. Note that a pairwise comparison of FISH and ISH was not possible for all cases, due to incomplete overlap of cases analysed with each assay.

Table 5 Nuclear/cytoplasmic localisation of c-Myc comparing normal and invasive cells

Normal cells (frequency percent)	**Invasive cells (frequency percent)**		**Total**
(A) Nuclear localisation			
	–	+	
–	28	4	32
+	14	8	22
Total	42	12	54
(B) Cytoplasmic localisation			
–	12	13	25
+	9	20	21
Total	21	33	54

In all, 54 pairs (normal vs invasive) of tissues were analysed to answer the questions of (1) whether positivity of nuclear cells in normal tissues is different from that in invasive cells, and similarly (2) whether positivity of cytoplasmic cells in normal tissues is different from that in invasive cells. The data are summarised in the above contingency tables. In all, 22 normal cell specimens were positive for c-Myc staining (40.71%), compared to 12 specimens (22.2%) in invasive cells. The difference is statistically significant ($P=0.01$) by McNemar's χ^2 test (two-sided).

Table 6 Correlation between c-Myc protein expression (IHC) and c-*myc* gene copy number (FISH)

	FISH	
IHC (% cells)	**Low**	**High**
Low	3	0
High	10	15
		$P=0.0016$

Consecutive serial sections of high-grade human breast tumours were scored for c-*myc* gene copy number or protein expression, by immunohistochemistry (IHC). IHC scores were defined in the Materials and methods section. Data were analysed for correlations between the results. A highly significant correlation was observed between high c-Myc protein expression (IHC) between percent cells positive and high c-*myc* gene amplification (FISH). $P=0.0016$ from two-sided McNemar's test. Note that for 15 cases, no staining for c-Myc could be detected; these negative cases were not included in the correlation presented, above.

Table 4 c-Myc immunohistochemistry (IHC) results

Staining intensity	**0**	**1**	**2**	**3**	**Percent positivity**	**1**	**2**	**3**	**4**
Number of tumour samples in each category	15	13	20	3	Number of tumour samples in each category	2	2	1	29

In all, 51 high-grade human breast carcinomas were analysed to determine the relationships between c-Myc protein expression and c-*myc* gene *in situ* hybridisation results. Data are shown in two ways in the above table. First, overall staining intensity of c-Myc-positive cells was scored as 0, 1, 2, 3 (low to high), and the number of tumour samples at each level of staining is indicated on the line below. Next, in a random subset of these cases, the percentage of tumour cells staining was scored as 0, 1, 2, 3, 4 (low to high %, as discussed in Materials and methods). The number of tumours at each level of percent cell positivity for c-Myc is indicated on the line below.

densitometry, after Western blot. These data showed c-*myc* gene amplification in 21% of tumours (Jenkins *et al*, 1997), using assays not based on *in situ* discrimination of tumour *vs* nontumour cells. The lower frequency of c-*myc* in this prior study is in contrast with the data we present here, and could be the result of the higher sensitivity and precision of the FISH and immunohistochemical methods, as distinct from quantitative PCR and Western blot densitometry. In addition, the 70% of amplified tumours in our study is also much higher than the 12% reported by Schraml *et al* (1999), using a c-*myc* FISH test on a tissue microarray. This large difference may be because the arrays are prepared from cores of paraffin-embedded tissue, as small as 0.6 mm in diameter which may contain too few tumour cells for complete analysis of amplification of a gene, such as c-*myc*. c-*myc* is known to be quite heterogeneous in its gene amplification within individual tumours (in contrast to *HER2/neu*, for example) (Persons *et al*, 1997).

Most previous reports on the expression of c-*myc* mRNA have utilised Northern blot, dot blot or PCR-based approaches, while just a few involved *in situ* hybridisation, which were primarily performed on frozen tissue sections (Liao and Dickson, 2000). Normal breast tissue is dominated by adipose cells, differing greatly from tumour tissue in its epithelial cellularity. Thus, normal and tumour tissues may not be rigorously compared by techniques involving RNA extraction from total tissue. Therefore, conclusions such as 'increased expression' may be more difficult to make from studies with Northern blot, dot blot and PCR-based techniques that require RNA extraction from tissues that have not been fastidiously micro-dissected for selection of tumour cells. Using a more sensitive, nonradioactive *in situ* hybridisation (ISH) approach on formalin-fixed, paraffin-embedded sections, we report herein high expression of c-*myc* mRNA in 92% of high-grade breast carcinomas. This figure is much higher than the recently reported data (22%), obtained by using a real-time RT-PCR method (Bieche *et al*, 1999). Dilution of the RNA from epithelium by the RNA from adipose in normal breast tissue in this latest prior report may be one of the possible explanations for this large difference.

In conclusion, the present study shows that approximately 70, 92 and 70% of biopsies of untreated high-grade breast cancer exhibit c-*myc* gene amplification, mRNA overexpression and protein overexpression, respectively. In most cases (84%), with gene copy amplification, the c-*myc* gene gains one to two additional copies. c-*myc* gene amplification was significantly associated with expression of its mRNA (both by intensity in invasive cells and by percentage positivity in invasive cells), and with expression of its protein (by percentage positivity in invasive cells). However, our data were also consistent with the prior literature on c-Myc (reviewed in Nass and Dickson, 1997; Liao and Dickson, 2000), indicating complex transcriptional, post transcriptional, translational and post-translational control of c-Myc expression *in vitro*. Specifically, in Table 5 we observed that in 40% of the high-grade tumours tested, c-Myc protein was expressed at high levels, despite a lack of its gene amplification.

It will be interesting to analyse lower grade tumours and premalignant lesions, with the same measurement tools, to determine if this c-*myc* amplification pattern is different, comparing different steps in onset and progression of the disease. Specifically, prior studies in fibroblasts and in human mammary epithelial cells (Liao *et al*, 1998, 2000a, b) have demonstrated that only a subtle deregulation of expression of c-Myc is sufficient to allow genomic instability. These prior cell biologic findings raise the question of whether c-Myc protein expression precedes or follows its gene amplification during the course of the natural history of breast cancer. It will also be interesting for future studies of lower grade breast cancers and premalignant lesions to determine whether there is evidence of nuclear exclusion of c-Myc protein. Indeed, nuclear exclusion of c-Myc in high-grade tumours could serve to attenuate its functions in later stages of disease progression (Liao and Dickson, 2000).

ACKNOWLEDGEMENTS

We would like to express our gratitude to Mr G Veytsman and H-K Cao for scoring FISH data, Mrs S Constable for helping in tissue sectioning. This work was supported by NIH Grants RO1 CA72460 and AG1496 to RB Dickson and by a pilot grant from the Georgetown University ACS Institutional Research Grant to JK Blancato. This work was supported by NIH Grants RO1 CA72460 and AG1496 to RB Dickson and by an ACS pilot grant (from the GU Institutional ACS Research Grant) to JK Blancato.

REFERENCES

Augenlich L, Wadler S, Gorner G, Richards C, Ryan L, Multani A, Pathak S, Benson A, Haller D, Heerdt B (1997) Low-level c-*myc* amplification in human colonic carcinoma cell lines and tumors: a frequent, p53-independent mutation associated with improved outcome in a randomized multi-institutional trial. *Cancer Res* 57: 1769–1775

Aulmann S, Bentz M, Sinn HP (2002) C-myc oncogene amplification in ductal carcinoma *in situ* of the breast. *Breast Cancer Res Treat* 74: 25–31

Berns E, Klijn J, Van Putten W, van Staveren I, Portengen H, Foekens J (1992) c-*myc* amplification is a better prognostic factor than *HER2/neu* amplification in primary breast cancer. *Cancer Res* 52: 1107–1113

Bieche I, Laurendeau I, Tozlu S, Olivi M, Vidaud D, Lidereau R, Vidaud M (1999) Quantitation of MYC gene expression in sporadic breast tumors with a real-time transcription-PCR assay. *Cancer Res* 59: 2759–2765

Chana J, Cree I, Foss A, Hungerford J, Wilson G (1998a) The prognositc significance of c-*myc* oncogene expression in uveal melanoma. *Melanoma Res* 8: 139–144

Chana J, Grover R, Wilson G, Hudson D, Forders M, Sanders R, Grobbelaar A (1998b) The clinical significance of c-*myc* oncogene expression in melanomas of the scalp. *Br J Plast Surg* 51: 191–194

Chana J, Wilson G, Cree I, Alexander R, Myatt N, Neale M, Foss A, Hungerford J (1999) c-*myc*, p53, and *bcl-2* expression and clinical outcome in uveal melanoma. *Br J Opthamol* 83: 110–114

Deming S, Nass S, Dickson R, Trock B (2000) c-*myc* amplification in breast cancer: a meta-analysis of its occurrence and prognostic relevance. *Br J Cancer* 83: 1688–1695

Diebold J, Suchy B, Baretton G, Blasenbreu S, Meier W, Schmidt M, Rabes H, Lohrs U (1996) DNA ploidy and MYC DNA amplification in ovarian carcinomas: correlation with p53 and bcl-2 expression, proliferative activity and prognosis. *Virchows Arch* 429: 221–227

Grover R, Chana J, Grobbelaar A, Hudson D, Forders M, Wilson G, Sanders R (1999) Measurement of c-*myc* oncogene expression provides an accurate prognostic marker for acral lentiginous melanoma. *Br J Plast Surg* 52: 122–126

Grover R, Ross D, Wilson G, Sanders R (1997) Measurement of c-*myc* oncoprotein provides an independent prognostic marker for regional metastatic melanoma. *Br J Plast Surg* 50: 478–482

Hyman E, Kauraniemi P, Hautaniemi S, Wolf M, Mousses S, Rozenblum E, Ringner M, Sauter G, Monni O, Elkahloun A, Kallioniemi O, Kallioniemi A (2002) Impact of DNA amplification on gene expression patterns in breast cancer. *Cancer Res* 62: 6240–6245

Isola J, Chu L, De Vries S, Matsumura K, Chew K, Ljung B, Waldman F (1999) Genetic alterations in ERBB2-amplified breast carcinomas. *Clin Cancer Res* 5: 4140–4145

Jenkins R, Qian J, Lieber M, Bostwick D (1997) Detection of c-*myc* oncogene amplification and chromosomal anomalies in metastatic

prostatic carcinoma by fluorescence *in situ* hybridization. *Cancer Res* **57**: 524–531

Liao D, Dickson R (2000) c-Myc in breast cancer. *Endocrine-Relat Cancer* **7**: 143–164

Liao D, Hou X, Bai S, Li S, Li J (2000a) Unusual deregulation of cell cycle components in early and frank estrogen-induced renal neoplasias in the Syrian hamster. *Carcinogenesis* **21**: 2167–2173

Liao D, Natarajan G, Deming S, Jamerson M, Johnson M, Chepko G, Dickson R (2000b) Cell cycle basis for the onset and progression of c-Myc-induced, TGFa-enhanced mouse mammary gland carcinogenesis. *Oncogene* **19**: 1307–1317

Liao D, Pantazis C, Hou X, Li S (1998) Promotion of estrogen-induced mammary gland carcinogenesis by androgen in the male Noble rat: probable mediation by steroid receptors. *Carcinogenesis* **19**: 2173–2180

Marcu K, Bossone S, Patel A (1992) Myc function and regulation. *Annu Rev Biochem* **61**: 809–860

Melhem M, Meisler A, Finley G, Bryce W, Jones M, Tribby I, Pipas J, Koski R (1992) Distribution of cells expressing Myc proteins in human colorectal epithelium, polyps, and malignant tumors. *Cancer Res* **52**: 5853–5864

Nass S, Dickson R (1997) Defining a role for c-Myc in breast tumorigenesis. *Breast Cancer Res Treat* **44**: 1–22

Nesbit C, Tersak J, Prochownik E (1999) MYC oncogenes and human neoplastic disease. *Oncogene* **18**: 3004–3016

Persons D, Borelli K, Hsu P (1997) Quantitation of *HER-2/neu* and c-*myc* gene amplification in breast carcinoma using fluorescence *in situ* hybridization. *Mod Pathol* **10**: 720–727

Pietilainen T, Lipponen P, Aaltomaa S, Eskelinen M, Kosma V, Syrjanen K (1995) Expression of c-*myc* proteins in breast cancer as related to established prognostic factors and survival. *Anticancer Res* **15**: 959–964

Polacarz S, Hey N, Stephenson T, Hill A (1989) c-*myc* oncogene product $p62^{c\text{-}myc}$ in ovarian mucinous neoplasms: immunohistochemical study correlated with malignancy. *J Clin Pathol* **42**: 148–152

Pollack JR, Sorlie T, Perou CM, Rees CA, Jeffrey SS, Lonning PE, Tibshirani R, Botstein D, Borresen-Dale AL, Brown PO (2002) Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors. *Proc Natl Acad Sci USA* **99**: 12963–12968

Sartelet S, Grossi L, Pasquier D, Conbaret V, Bouvier R, Ranchere D, Plantaz D, Munzer M, Philip T, Birembaut P, Zahm J, Bergeron C, Gaillard D, Pasquier B (2002) Detection of N-myc amplification by FISH in immature areas of fixed neuroblastomas: more efficient than Southern blot/PCR. *J Pathol* **198**: 83–91

Sasano H, Nagura H, Silverberg S (1992) Immunolocalization of c-*myc* oncoprotein in mucinous and serous adenocarcinomas of the ovary. *Hum Pathol* **23**: 491–495

Sato H, Tsuchiya A, Abe R (1995) Correlation between c-*myc* protein expression and phases of the cell cycle in human colorectal carcinomas. *Fukushima J Med Sci* **41**: 111–113

Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Noctito A, Mihatsch M, Kallioniemi O, Sauter G (1999) Tissue microarrays for gene amplification surveys in many different tumor types. *Clin Cancer Res* **5**: 1966–1975

Sikora K, Chan S, Evan G, Gabra H, Markham N, Stewart J, Watson J (1987) c-*myc* oncogene expression in colorectal cancer. *Cancer* **59**: 1289–1295

Sikora K, Evan G, Stewart J, Watson J (1985) Detection of the c-*myc* oncogene product in testicular cancer. *Br J Cancer* **52**: 171–176

Smith D, Goh H-S (1996) Overexpression of c-*myc* proto-oncogene in colorectal carcinoma is associated with a reduced mortality that is abrogated by point mutation of the p53 tumor suppressor gene. *Clin Cancer Res* **2**: 1049–1053

Sundaresan V, Forgacs I, Wight D, Wilson B, Evan G, Watson J (1987) Abnormal distribution of c-*myc* oncogene product in familial adenomatous polyposis. *J Clin Pathol* **40**: 1274–1281

Visca P, Alo P, Del Nonno F, Bott C, Trombetta G, Marandino F, Filippi S, Di Tondo U, Donnorso R (1999) Immunohistochemical expression of fatty acid synthase, apoptotic-regulating genes, proliferating factors, and *ras*, protein product in colorectal adenomas, carcinomas, and adjacent nonneoplastic mucosa. *Clin Cancer Res* **5**: 4111–4118

Visscher D, Wallis T, Awussah S, Mohamed A, Crissman J (1997) Evaluation of MYC and chromosome 8 copy number in breast carcinoma by interphase cytogenetics. *Genes, Chromosomes, Cancer* **6**: 389–404

Voravud N, Foster C, Gilbertson J, Sikora K, Waxman J (1989) Oncogene expression in cholangiocarcinoma and in normal hepatic development. *Hum Pathol* **20**: 1163–1168

Watson J, Stewart J, Evan G, Ritson A, Sikora K (1986) The clinical significance of low cytometric c-*myc* oncoprotein in testicular cancer. *Br J Cancer* **53**: 331–337

Williams A, Piris J, Wyllie A (1990) Immunohistochemical demonstration of altered intracellular localization of the c-*myc* oncogene product in human colorectal neoplasms. *J Pathol* **160**: 287–293

Yuen M, Wu P, Lau J, Lai C (2001) Expression of c-Myc, c-Fos, and c-Jun in hepatocellular carcinoma. *Cancer* **91**: 106–112

Zar JH (1974) *Biostatistical Analysis*. Prentice-Hall: Englewood Cliffs, NJ

MOLECULAR BIOLOGY OF THE CELL

fourth edition

Bruce Alberts

Alexander Johnson

Julian Lewis

Martin Raff

Keith Roberts

Peter Walter

GS Garland Science
Taylor & Francis Group

Garland
Vice President: Denise Schanck
Managing Editor: Sarah Gibbs
Senior Editorial Assistant: Kirsten Jenner
Managing Production Editor: Emma Hunt
Proofreader and Layout: Emma Hunt
Production Assistant: Angela Bennett
Text Editors: Marjorie Singer Anderson and Betsy Dilernia
Copy Editor: Bruce Goatly
Word Processors: Fran Dependahl, Misty Landers and Carol Winter
Designer: Blink Studio, London
Illustrator: Nigel Orme
Indexer: Janine Ross and Sherry Granum
Manufacturing: Nigel Eyre and Marion Morrow

Bruce Alberts received his Ph.D. from Harvard University and is President of the National Academy of Sciences and Professor of Biochemistry and Biophysics at the University of California, San Francisco. **Alexander Johnson** received his Ph.D. from Harvard University and is a Professor of Microbiology and Immunology at the University of California, San Francisco. **Julian Lewis** received his D.Phil. from the University of Oxford and is a Principal Scientist at the Imperial Cancer Research Fund, London. **Martin Raff** received his M.D. from McGill University and is at the Medical Research Council Laboratory for Molecular Cell Biology and Cell Biology Unit and in the Biology Department at University College London. **Keith Roberts** received his Ph.D. from the University of Cambridge and is Associate Research Director at the John Innes Centre, Norwich. **Peter Walter** received his Ph.D. from The Rockefeller University in New York and is Professor and Chairman of the Department of Biochemistry and Biophysics at the University of California, San Francisco, and an Investigator of the Howard Hughes Medical Institute.

Library of Congress Cataloging-in-Publicaton Data
Molecular biology of the cell / Bruce Alberts ... [et al.].-- 4th ed.
p. cm
Includes bibliographical references and index.
ISBN 0-8153-3218-1 (hardbound) -- ISBN 0-8153-4072-9 (pbk.)
1. Cytology. 2. Molecular biology. I. Alberts, Bruce.
[DNLM: 1. Cells. 2. Molecular Biology.]
QH581.2 .M64 2002
571.6--dc21

2001054471 CIP

Published by Garland Science, a member of the Taylor & Francis Group, 29 West 35th Street, New York, NY 10001-2299

Printed in the United States of America

15 14 13 12 11 10 9 8 7 6 5 4 3 2 1

Cell Biology Interactive
Artistic and Scientific Direction: Peter Walter
Narrated by: Julie Theriot
Production, Design, and Development: Mike Morales

Front cover Human Genome: Reprinted by permission from *Nature*, International Human Genome Sequencing Consortium, 409:860–921, 2001 © Macmillan Magazines Ltd. Adapted from an image by Francis Collins, NHGRI; Jim Kent, UCSC; Ewan Birney, EBI; and Darryl Leja, NHGRI; showing a portion of Chromosome 1 from the initial sequencing of the human genome.

Back cover In 1967, the British artist Peter Blake created a design classic. Nearly 35 years later Nigel Orme (illustrator), Richard Denyer (photographer), and the authors have together produced an affectionate tribute to Mr Blake's image. With its gallery of icons and influences, its assembly created almost as much complexity, intrigue and mystery as the original. *Drosophila, Arabidopsis,* Dolly and the assembled company tempt you to dip inside where, as in the original, "a splendid time is guaranteed for all." (Gunter Blobel, courtesy of The Rockefeller University; Marie Curie, Keystone Press Agency Inc; Darwin bust, by permission of the President and Council of the Royal Society; Rosalind Franklin, courtesy of Cold Spring Harbor Laboratory Archives; Dorothy Hodgkin, © The Nobel Foundation, 1964; James Joyce, etching by Peter Blake; Robert Johnson, photo booth self-portrait early 1930s, © 1986 Delta Haze Corporation all rights reserved, used by permission; Albert L. Lehninger, (unidentified photographer) courtesy of The Alan Mason Chesney Medical Archives of The Johns Hopkins Medical Institutions; Linus Pauling, from Ava Helen and Linus Pauling Papers, Special Collections, Oregon State University; Nicholas Poussin, courtesy of ArtToday.com; Barbara McClintock, © David Micklos, 1983; Andrei Sakharov, courtesy of Elena Bonner; Frederick Sanger, © The Nobel Foundation, 1958.)

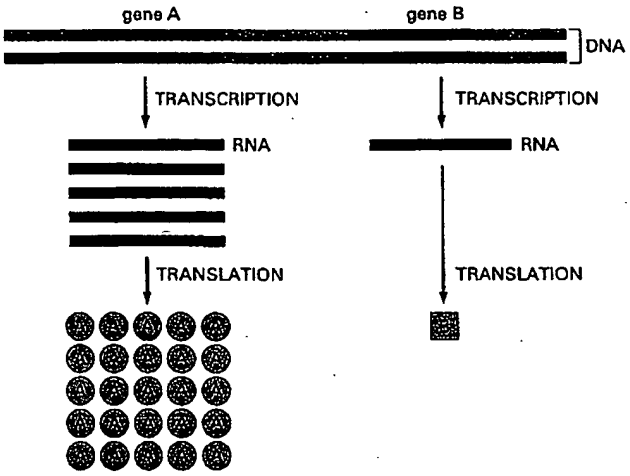


Figure 6–3 Genes can be expressed with different efficiencies. Gene A is transcribed and translated much more efficiently than gene B. This allows the amount of protein A in the cell to be much greater than that of protein B.

FROM DNA TO RNA

Transcription and translation are the means by which cells read out, or express, the genetic instructions in their genes. Because many identical RNA copies can be made from the same gene, and each RNA molecule can direct the synthesis of many identical protein molecules, cells can synthesize a large amount of protein rapidly when necessary. But each gene can also be transcribed and translated with a different efficiency, allowing the cell to make vast quantities of some proteins and tiny quantities of others (Figure 6–3). Moreover, as we see in the next chapter, a cell can change (or regulate) the expression of each of its genes according to the needs of the moment—most obviously by controlling the production of its RNA.

Portions of DNA Sequence Are Transcribed into RNA

The first step a cell takes in reading out a needed part of its genetic instructions is to copy a particular portion of its DNA nucleotide sequence—a gene—into an RNA nucleotide sequence. The information in RNA, although copied into another chemical form, is still written in essentially the same language as it is in DNA—the language of a nucleotide sequence. Hence the name **transcription.**

Like DNA, RNA is a linear polymer made of four different types of nucleotide subunits linked together by phosphodiester bonds (Figure 6–4). It differs from DNA chemically in two respects: (1) the nucleotides in RNA are *ribonucleotides*—that is, they contain the sugar ribose (hence the name *ribo*nucleic acid) rather than deoxyribose; (2) although, like DNA, RNA contains the bases adenine (A), guanine (G), and cytosine (C), it contains the base uracil (U) instead of the thymine (T) in DNA. Since U, like T, can base-pair by hydrogen-bonding with A (Figure 6–5), the complementary base-pairing properties described for DNA in Chapters 4 and 5 apply also to RNA (in RNA, G pairs with C, and A pairs with U). It is not uncommon, however, to find other types of base pairs in RNA: for example, G pairing with U occasionally.

Despite these small chemical differences, DNA and RNA differ quite dramatically in overall structure. Whereas DNA always occurs in cells as a double-stranded helix, RNA is single-stranded. RNA chains therefore fold up into a variety of shapes, just as a polypeptide chain folds up to form the final shape of a protein (Figure 6–6). As we see later in this chapter, the ability to fold into complex three-dimensional shapes allows some RNA molecules to have structural and catalytic functions.

Transcription Produces RNA Complementary to One Strand of DNA

All of the RNA in a cell is made by DNA transcription, a process that has certain similarities to the process of DNA replication discussed in Chapter 5.

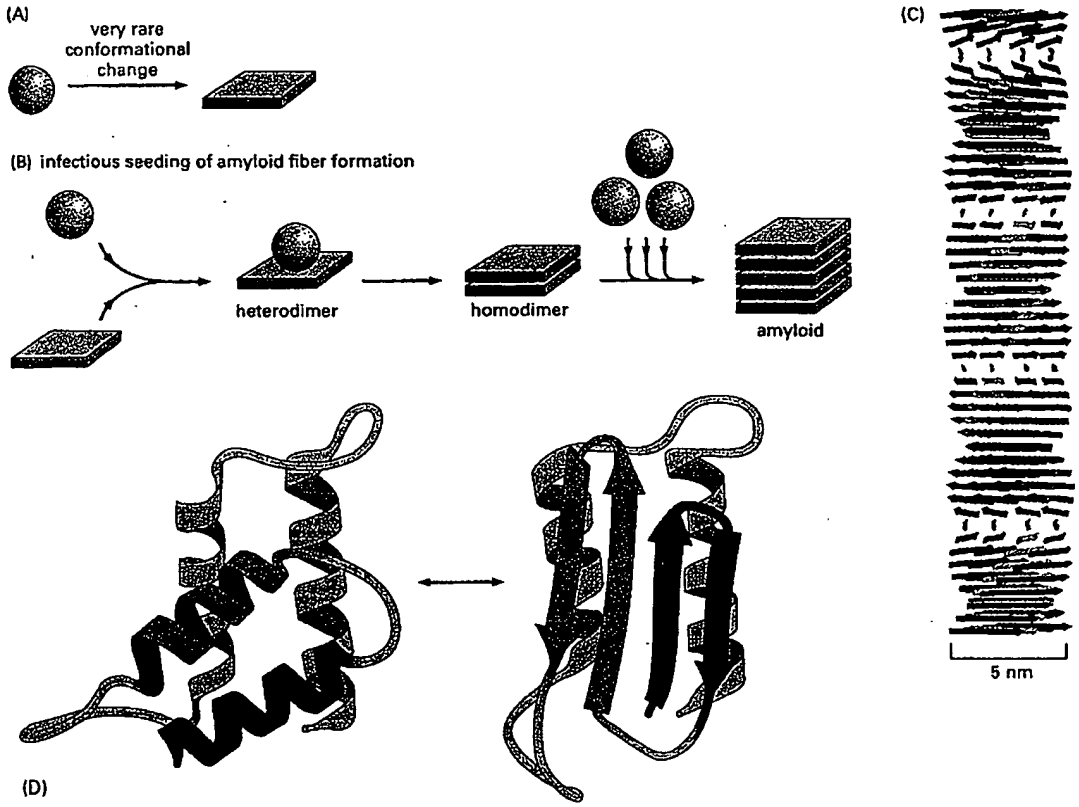


Figure 6–89 Protein aggregates that cause human disease. (A) Schematic illustration of the type of conformational change in a protein that produces material for a cross-beta filament. (B) Diagram illustrating the self-infectious nature of the protein aggregation that is central to prion diseases. PrP is highly unusual because the misfolded version of the protein, called PrP*, induces the normal PrP protein it contacts to change its conformation, as shown. Most of the human diseases caused by protein aggregation are caused by the overproduction of a variant protein that is especially prone to aggregation, but because this structure is not infectious in this way, it cannot spread from one animal to another. (C) Drawing of a cross-beta filament, a common type of protease-resistant protein aggregate found in a variety of human neurological diseases. Because the hydrogen-bond interactions in a β sheet form between polypeptide backbone atoms (see Figure 3–9), a number of different abnormally folded proteins can produce this structure. (D) One of several possible models for the conversion of PrP to PrP*, showing the likely change of two α-helices into four β-strands. Although the structure of the normal protein has been determined accurately, the structure of the infectious form is not yet known with certainty because the aggregation has prevented the use of standard structural techniques. (C, courtesy of Louise Serpell, adapted from M. Sunde et al., *J. Mol. Biol.* 273:729–739, 1997; D, adapted from S.B. Prusiner, *Trends Biochem. Sci.* 21:482–487, 1996.)

animals and humans. It can be dangerous to eat the tissues of animals that contain PrP*, as witnessed most recently by the spread of BSE (commonly referred to as the "mad cow disease") from cattle to humans in Great Britain.

Fortunately, in the absence of PrP*, PrP is extraordinarily difficult to convert to its abnormal form. Although very few proteins have the potential to misfold into an infectious conformation, a similar transformation has been discovered to be the cause of an otherwise mysterious "protein-only inheritance" observed in yeast cells.

There Are Many Steps From DNA to Protein

We have seen so far in this chapter that many different types of chemical reactions are required to produce a properly folded protein from the information contained in a gene (Figure 6–90). The final level of a properly folded protein in a cell therefore depends upon the efficiency with which each of the many steps is performed.

We discuss in Chapter 7 that cells have the ability to change the levels of their proteins according to their needs. In principle, any or all of the steps in Fig-

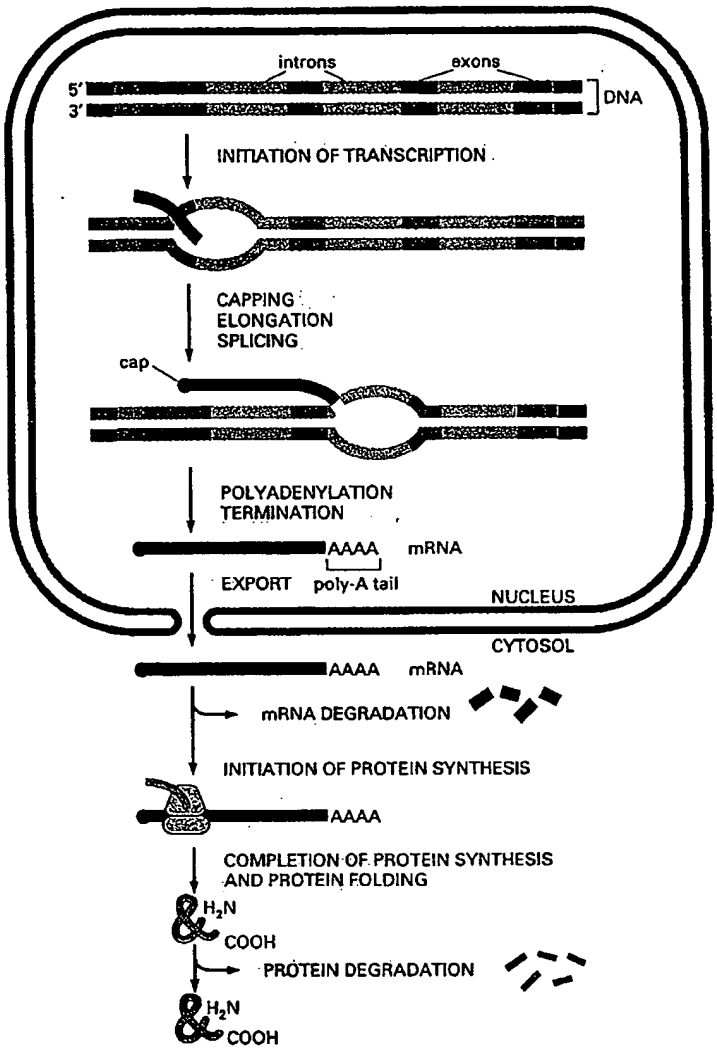


Figure 6–90 The production of a protein by a eucaryotic cell. The final level of each protein in a eucaryotic cell depends upon the efficiency of each step depicted.

ure 6–90) could be regulated by the cell for each individual protein. However, as we shall see in Chapter 7, the initiation of transcription is the most common point for a cell to regulate the expression of each of its genes. This makes sense, inasmuch as the most efficient way to keep a gene from being expressed is to block the very first step—the transcription of its DNA sequence into an RNA molecule.

Summary

The translation of the nucleotide sequence of an mRNA molecule into protein takes place in the cytoplasm on a large ribonucleoprotein assembly called a ribosome. The amino acids used for protein synthesis are first attached to a family of tRNA molecules, each of which recognizes, by complementary base-pair interactions, particular sets of three nucleotides in the mRNA (codons). The sequence of nucleotides in the mRNA is then read from one end to the other in sets of three according to the genetic code.

To initiate translation, a small ribosomal subunit binds to the mRNA molecule at a start codon (AUG) that is recognized by a unique initiator tRNA molecule. A large ribosomal subunit binds to complete the ribosome and begin the elongation phase of protein synthesis. During this phase, aminoacyl tRNAs—each bearing a specific amino acid bind sequentially to the appropriate codon in mRNA by forming complementary base pairs with the tRNA anticodon. Each amino acid is added to the C-terminal end of the growing polypeptide by means of a cycle of three sequential

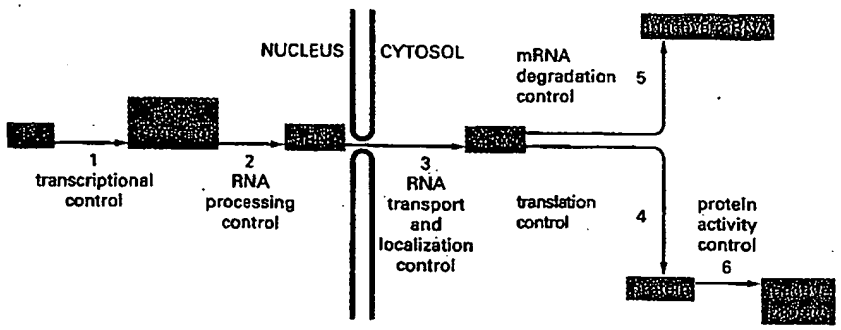


Figure 7–5 Six steps at which eucaryotic gene expression can be controlled. Controls that operate at steps 1 through 5 are discussed in this chapter. Step 6, the regulation of protein activity, includes reversible activation or inactivation by protein phosphorylation (discussed in Chapter 3) as well as irreversible inactivation by proteolytic degradation (discussed in Chapter 6).

Gene Expression Can Be Regulated at Many of the Steps in the Pathway from DNA to RNA to Protein

If differences among the various cell types of an organism depend on the particular genes that the cells express, at what level is the control of gene expression exercised? As we saw in the last chapter, there are many steps in the pathway leading from DNA to protein, and all of them can in principle be regulated. Thus a cell can control the proteins it makes by (1) controlling when and how often a given gene is transcribed (**transcriptional control**), (2) controlling how the RNA transcript is spliced or otherwise processed (**RNA processing control**), (3) selecting which completed mRNAs in the cell nucleus are exported to the cytosol and determining where in the cytosol they are localized (**RNA transport and localization control**), (4) selecting which mRNAs in the cytoplasm are translated by ribosomes (**translational control**), (5) selectively destabilizing certain mRNA molecules in the cytoplasm (**mRNA degradation control**), or (6) selectively activating, inactivating, degrading, or compartmentalizing specific protein molecules after they have been made (**protein activity control**) (Figure 7–5).

For most genes transcriptional controls are paramount. This makes sense because, of all the possible control points illustrated in Figure 7–5, only transcriptional control ensures that the cell will not synthesize superfluous intermediates. In the following sections we discuss the DNA and protein components that perform this function by regulating the initiation of gene transcription. We shall return at the end of the chapter to the additional ways of regulating gene expression.

Summary

The genome of a cell contains in its DNA sequence the information to make many thousands of different protein and RNA molecules. A cell typically expresses only a fraction of its genes, and the different types of cells in multicellular organisms arise because different sets of genes are expressed. Moreover, cells can change the pattern of genes they express in response to changes in their environment, such as signals from other cells. Although all of the steps involved in expressing a gene can in principle be regulated, for most genes the initiation of RNA transcription is the most important point of control.

DNA-BINDING MOTIFS IN GENE REGULATORY PROTEINS

How does a cell determine which of its thousands of genes to transcribe? As mentioned briefly in Chapters 4 and 6, the transcription of each gene is controlled by a regulatory region of DNA relatively near the site where transcription begins. Some regulatory regions are simple and act as switches that are thrown by a single signal. Many others are complex and act as tiny microprocessors, responding to a variety of signals that they interpret and integrate to switch the neighboring gene on or off. Whether complex or simple, these switching devices

occur in the germ line, the cell lineage that gives rise to sperm or eggs. Most of the DNA in vertebrate germ cells is inactive and highly methylated. Over long periods of evolutionary time, the methylated CG sequences in these inactive regions have presumably been lost through spontaneous deamination events that were not properly repaired. However promoters of genes that remain active in the germ cell lineages (including most housekeeping genes) are kept unmethylated, and therefore spontaneous deaminations of Cs that occur within them can be accurately repaired. Such regions are preserved in modern day vertebrate cells as CG islands. In addition, any mutation of a CG sequence in the genome that destroyed the function or regulation of a gene in the adult would be selected against, and some CG islands are simply the result of a higher than normal density of critical CG sequences.

The mammalian genome contains an estimated 20,000 CG islands. Most of the islands mark the 5′ ends of transcription units and thus, presumably, of genes. The presence of CG islands often provides a convenient way of identifying genes in the DNA sequences of vertebrate genomes.

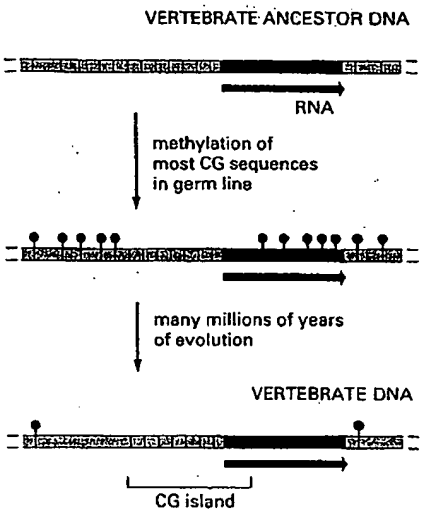


Figure 7–86 A mechanism to explain both the marked overall deficiency of CG sequences and their clustering into CG islands in vertebrate genomes. A *black line* marks the location of a CG dinucleotide in the DNA sequence, while a *red* "lollipop" indicates the presence of a methyl group on the CG dinucleotide. CG sequences that lie in regulatory sequences of genes that are transcribed in germ cells are unmethylated and therefore tend to be retained in evolution. Methylated CG sequences, on the other hand, tend to be lost through deamination of 5-methyl C to T, unless the CG sequence is critical for survival.

Summary

The many types of cells in animals and plants are created largely through mechanisms that cause different genes to be transcribed in different cells. Since many specialized animal cells can maintain their unique character through many cell division cycles and even when grown in culture, the gene regulatory mechanisms involved in creating them must be stable once established and heritable when the cell divides. These features endow the cell with a memory of its developmental history. Bacteria and yeasts provide unusually accessible model systems in which to study gene regulatory mechanisms. One such mechanism involves a competitive interaction between two gene regulatory proteins, each of which inhibits the synthesis of the other; this can create a flip-flop switch that switches a cell between two alternative patterns of gene expression. Direct or indirect positive feedback loops, which enable gene regulatory proteins to perpetuate their own synthesis, provide a general mechanism for cell memory. Negative feedback loops with programmed delays form the basis for cellular clocks.

In eucaryotes the transcription of a gene is generally controlled by combinations of gene regulatory proteins. It is thought that each type of cell in a higher eucaryotic organism contains a specific combination of gene regulatory proteins that ensures the expression of only those genes appropriate to that type of cell. A given gene regulatory protein may be active in a variety of circumstances and typically is involved in the regulation of many genes.

In addition to diffusible gene regulatory proteins, inherited states of chromatin condensation are also used by eucaryotic cells to regulate gene expression. An especially dramatic case is the inactivation of an entire X chromosome in female mammals. In vertebrates DNA methylation also functions in gene regulation, being used mainly as a device to reinforce decisions about gene expression that are made initially by other mechanisms. DNA methylation also underlies the phenomenon of genomic imprinting in mammals, in which the expression of a gene depends on whether it was inherited from the mother or the father.

POSTTRANSCRIPTIONAL CONTROLS

In principle, every step required for the process of gene expression could be controlled. Indeed, one can find examples of each type of regulation, although any one gene is likely to use only a few of them. Controls on the initiation of gene transcription are the predominant form of regulation for most genes. But other controls can act later in the pathway from DNA to protein to modulate the amount of gene product that is made. Although these **posttranscriptional controls**, which operate after RNA polymerase has bound to the gene's promoter and begun RNA synthesis, are less common than *transcriptional control*, for many genes they are crucial.

Genome-wide Study of Gene Copy Numbers, Transcripts, and Protein Levels in Pairs of Non-invasive and Invasive Human Transitional Cell Carcinomas*

Torben F. Ørntoft‡§, Thomas Thykjaer¶, Frederic M. Waldman‖, Hans Wolf**, and Julio E. Celis‡‡

Gain and loss of chromosomal material is characteristic of bladder cancer, as well as malignant transformation in general. The consequences of these changes at both the transcription and translation levels is at present unknown partly because of technical limitations. Here we have attempted to address this question in pairs of non-invasive and invasive human bladder tumors using a combination of technology that included comparative genomic hybridization, high density oligonucleotide array-based monitoring of transcript levels (5600 genes), and high resolution two-dimensional gel electrophoresis. The results showed that there is a gene dosage effect that in some cases superimposes on other regulatory mechanisms. This effect depended ($p < 0.015$) on the magnitude of the comparative genomic hybridization change. In general (18 of 23 cases), chromosomal areas with more than 2-fold gain of DNA showed a corresponding increase in mRNA transcripts. Areas with loss of DNA, on the other hand, showed either reduced or unaltered transcript levels. Because most proteins resolved by two-dimensional gels are unknown it was only possible to compare mRNA and protein alterations in relatively few cases of well focused abundant proteins. With few exceptions we found a good correlation ($p < 0.005$) between transcript alterations and protein levels. The implications, as well as limitations, of the approach are discussed. *Molecular & Cellular Proteomics 1:37–45, 2002.*

Aneuploidy is a common feature of most human cancers (1), but little is known about the genome-wide effect of this phenomenon at both the transcription and translation levels. High throughput array studies of the breast cancer cell line BT474 has suggested that there is a correlation between DNA copy numbers and gene expression in highly amplified areas (2), and studies of individual genes in solid tumors have revealed a good correlation between gene dose and mRNA or protein levels in the case of c-erb-B2, *cyclin d1*, *ems1*, and N-myc (3–5). However, a high cyclin D1 protein expression has been observed without simultaneous amplification (4), and a low level of c-myc copy number increase was observed without concomitant c-myc protein overexpression (6).

In human bladder tumors, karyotyping, fluorescent *in situ* hybridization, and comparative genomic hybridization (CGH)[1] have revealed chromosomal aberrations that seem to be characteristic of certain stages of disease progression. In the case of non-invasive pTa transitional cell carcinomas (TCCs), this includes loss of chromosome 9 or parts of it, as well as loss of Y in males. In minimally invasive pT1 TCCs, the following alterations have been reported: 2q−, 11p−, 1q+, 11q13+, 17q+, and 20q+ (7–12). It has been suggested that these regions harbor tumor suppressor genes and oncogenes; however, the large chromosomal areas involved often contain many genes, making meaningful predictions of the functional consequences of losses and gains very difficult.

In this investigation we have combined genome-wide technology for detecting genomic gains and losses (CGH) with gene expression profiling techniques (microarrays and proteomics) to determine the effect of gene copy number on transcript and protein levels in pairs of non-invasive and invasive human bladder TCCs.

EXPERIMENTAL PROCEDURES

Material—Bladder tumor biopsies were sampled after informed consent was obtained and after removal of tissue for routine pathology examination. By light microscopy tumors 335 and 532 were staged by an experienced pathologist as pTa (superficial papillary),

From the ‡Department of Clinical Biochemistry, Molecular Diagnostic Laboratory and **Department of Urology, Aarhus University Hospital, Skejby, DK-8200 Aarhus N, Denmark, ¶AROS Applied Biotechnology ApS, Gustav Wiedsvej 10, DK-8000 Aarhus C, Denmark, ‖UCSF Cancer Center and Department of Laboratory Medicine, University of California, San Francisco, CA 94143-0808, and ‡‡Institute of Medical Biochemistry and Danish Centre for Human Genome Research, Ole Worms Allé 170, Aarhus University, DK-8000 Aarhus C, Denmark

Received, September 28, 2001, and in revised form, November 7, 2001

Published, MCP Papers in Press, November 13, 2001, DOI 10.1074/mcp.M100019-MCP200

[1] The abbreviations used are: CGH, comparative genomic hybridization; TCC, transitional cell carcinoma; LOH, loss of heterozygosity; PA-FABP, psoriasis-associated fatty acid-binding protein; 2D, two-dimensional.

FIG. 1. DNA copy number and mRNA expression level. Shown from *left* to *right* are chromosome (*Chr.*), CGH profiles, gene location and expression level of specific genes, and overall expression level along the chromosome. *A*, expression of mRNA in invasive tumor 733 as compared with the non-invasive counterpart tumor 335. *B*, expression of mRNA in invasive tumor 827 compared with the non-invasive counterpart tumor 532. The average fluorescent signal ratio between tumor DNA and normal DNA is shown along the length of the chromosome (*left*). The *bold curve* in the ratio profile represents a mean of four chromosomes and is surrounded by *thin curves* indicating one standard deviation. The *central vertical line* (*broken*) indicates a ratio value of 1 (no change), and the *vertical lines* next to it (*dotted*) indicate a ratio of 0.5 (*left*) and 2.0 (*right*). In chromosomes where the non-invasive tumor 335 used for comparison showed alterations in DNA content, the ratio profile of that chromosome is shown to the *right* of the invasive tumor profile. The *colored bars* represents one gene each, identified by the running *numbers above* the *bars* (the name of the gene can be seen at www.MDL.DK/sdata.html). The *bars* indicate the purported location of the gene, and the *colors* indicate the expression level of the gene in the invasive tumor compared with the non-invasive counterpart; >2-fold increase (*black*), >2-fold decrease (*blue*), no significant change (*orange*). The *bar* to the *far right*, entitled *Expression* shows the resulting change in expression along the chromosome; the colors indicate that at least half of the genes were up-regulated (*black*), at least half of the genes down-regulated (*blue*), or more than half of the genes are unchanged (*orange*). If a gene was absent in one of the samples and present in another, it was regarded as more than a 2-fold change. A 2-fold level was chosen as this corresponded to one standard deviation in a double determination of ~1800 genes. Centromeres and heterochromatic regions were excluded from data analysis.

grade I and II, respectively, tumors 733 and 827 were staged as pT1 (invasive into submucosa), 733 was staged as solid, and 827 was staged as papillary, both grade III.

mRNA Preparation—Tissue biopsies, obtained fresh from surgery, were embedded immediately in a sodium-guanidinium thiocyanate solution and stored at −80 °C. Total RNA was isolated using the RNAzol B RNA isolation method (WAK-Chemie Medical GMBH). poly(A)$^+$ RNA was isolated by an oligo(dT) selection step (Oligotex mRNA kit; Qiagen).

cRNA Preparation—1 μg of mRNA was used as starting material. The first and second strand cDNA synthesis was performed using the SuperScript® choice system (Invitrogen) according to the manufacturer's instructions but using an oligo(dT) primer containing a T7 RNA polymerase binding site. Labeled cRNA was prepared using the MEGAscript® *In vitro* transcription kit (Ambion). Biotin-labeled CTP and UTP (Enzo) was used, together with unlabeled NTPs in the reaction. Following the *In vitro* transcription reaction, the unincorporated nucleotides were removed using RNeasy columns (Qiagen).

Array Hybridization and Scanning—Array hybridization and scanning was modified from a previous method (13). 10 μg of cRNA was fragmented at 94 °C for 35 min in buffer containing 40 mM Tris acetate, pH 8.1, 100 mM KOAc, 30 mM MgOAc. Prior to hybridization, the fragmented cRNA in a 6× SSPE-T hybridization buffer (1 M NaCl, 10 mM Tris, pH 7.6, 0.005% Triton), was heated to 95 °C for 5 min, subsequently cooled to 40 °C, and loaded onto the Affymetrix probe array cartridge. The probe array was then incubated for 16 h at 40 °C at constant rotation (60 rpm). The probe array was exposed to 10 washes in 6× SSPE-T at 25 °C followed by 4 washes in 0.5× SSPE-T at 50 °C. The biotinylated cRNA was stained with a streptavidin-phycoerythrin conjugate, 10 μg/ml (Molecular Probes) in 6× SSPE-T

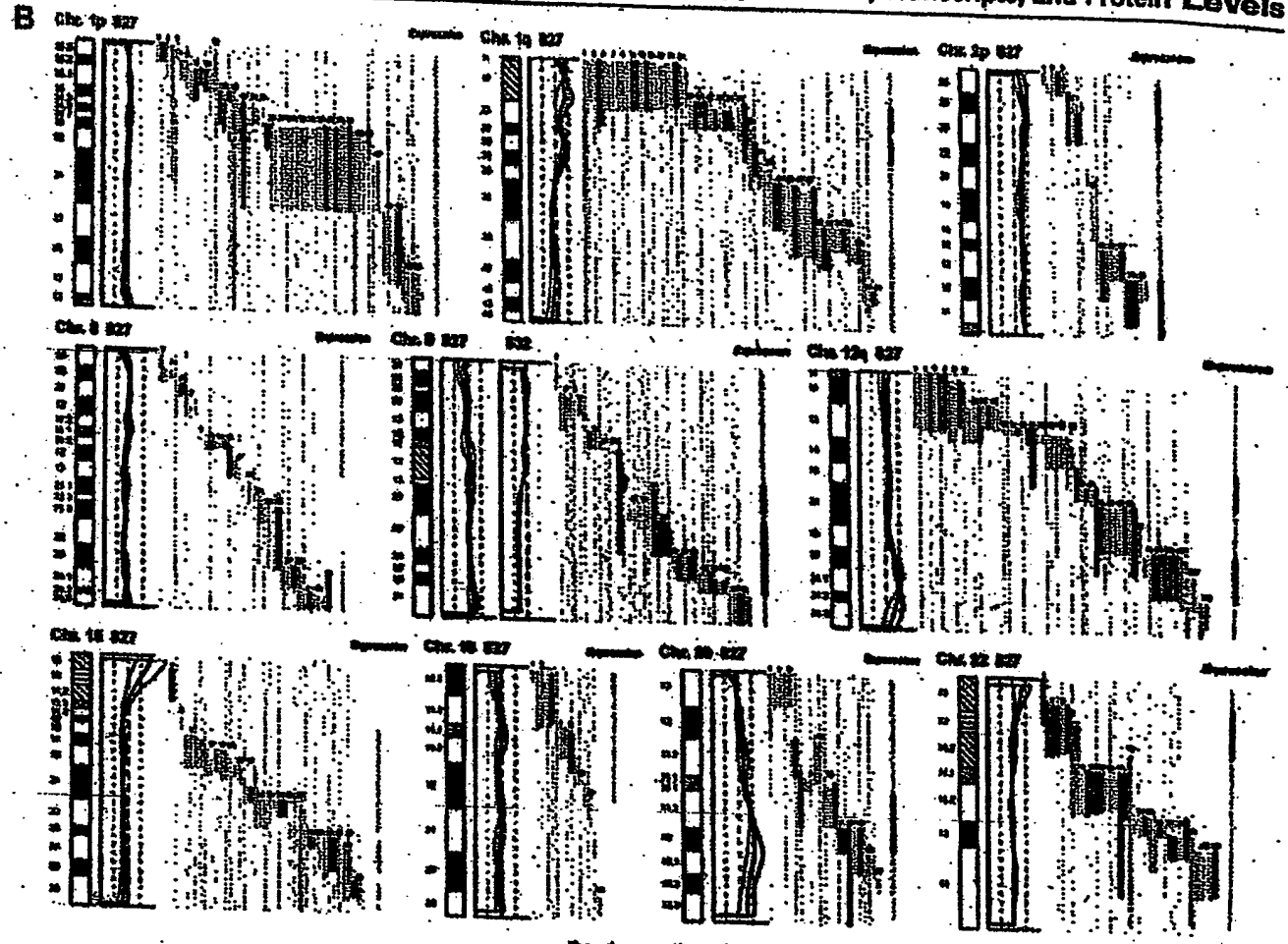


FIG. 1—*continued*

for 30 min at 25 °C followed by 10 washes in 6× SSPE-T at 25 °C. The probe arrays were scanned at 560 nm using a confocal laser scanning microscope (made for Affymetrix by Hewlett-Packard). The readings from the quantitative scanning were analyzed by Affymetrix gene expression analysis software.

Microsatellite Analysis—Microsatellite Analysis was performed as described previously (14). Microsatellites were selected by use of www.ncbi.nlm.nih.gov/genemap98, and primer sequences were obtained from the genome data base at www.gdb.org. DNA was extracted from tumor and blood and amplified by PCR in a volume of 20 μl for 35 cycles. The amplicons were denatured and electrophoresed for 3 h in an ABI Prism 377. Data were collected in the Gene Scan program for fragment analysis. Loss of heterozygosity was defined as less than 33% of one allele detected in tumor amplicons compared with blood.

Proteomic Analysis—TCCs were minced into small pieces and homogenized in a small glass homogenizer in 0.5 ml of lysis solution. Samples were stored at −20 °C until use. The procedure for 2D gel electrophoresis has been described in detail elsewhere (15, 16). Gels were stained with silver nitrate and/or Coomassie Brilliant Blue. Proteins were identified by a combination of procedures that included microsequencing, mass spectrometry, two-dimensional gel Western immunoblotting, and comparison with the master two-dimensional gel image of human keratinocyte proteins; see biobase.dk/cgi-bin/cells.

CGH—Hybridization of differentially labeled tumor and normal DNA to normal metaphase chromosomes was performed as described previously (10). Fluorescein-labeled tumor DNA (200 ng), Texas Red-labeled reference DNA (200 ng), and human Cot-1 DNA (20 μg) were denatured at 37 °C for 5 min and applied to denatured normal metaphase slides. Hybridization was at 37 °C for 2 days. After washing, the slides were counterstained with 0.15 μg/ml 4,6-diamidino-2-phenylindole in an anti-fade solution. A second hybridization was performed for all tumor samples using fluorescein-labeled reference DNA and Texas Red-labeled tumor DNA (inverse labeling) to confirm the aberrations detected during the initial hybridization. Each CGH experiment also included a normal control hybridization using fluorescein- and Texas Red-labeled normal DNA. Digital image analysis was used to identify chromosomal regions with abnormal fluorescence ratios, indicating regions of DNA gains and losses. The average green:red fluorescence intensity ratio profiles were calculated using four images of each chromosome (eight chromosomes total) with normalization of the green:red fluorescence intensity ratio for the entire metaphase and background correction. Chromosome identification was performed based on 4,6-diamidino-2-phenylindole banding patterns. Only images showing uniform high intensity fluorescence with minimal background staining were analyzed. All centromeres, p arms of acrocentric chromosomes, and heterochromatic regions were excluded from the analysis.

RESULTS

Comparative Genomic Hybridization—The CGH analysis identified a number of chromosomal gains and losses in the

TABLE I

Correlation between alterations detected by CGH and by expression monitoring

Top, CGH used as independent variable (# CGH alteration – what expression ratio was found); bottom, altered expression used as independent variable (# expression alteration – what CGH deviation was found).

CGH alterations	Tumor 733 vs. 335 Expression change clusters	Concordance	CGH alterations	Tumor 827 vs. 532 Expression change clusters	Concordance
13 Gain	10 Up-regulation 0 Down-regulation 3 No change	77%	10 Gain	8 Up-regulation 0 Down-regulation 2 No change	80%
10 Loss	1 Up-regulation 5 Down-regulation 4 No change	50%	12 Loss	3 Up-regulation 2 Down regulation 7 No change	17%

Expression change clusters	Tumor 733 vs. 335 CGH alterations	Concordance	Expression change clusters	Tumor 827 vs. 532 CGH alterations	Concordance
16 Up-regulation	11 Gain 2 Loss 3 No change	69%	17 Up-regulation	10 Gain 5 Loss 2 No change	59%
21 Down-regulation	1 Gain 8 Loss 12 No change	38%	9 Down-regulation	0 Gain 3 Loss 6 No change	33%
15 No change	3 Gain 3 Loss 9 No change	60%	21 No change	1 Gain 3 Loss 17 No change	81%

two invasive tumors (stage pT1, TCCs 733 and 827), whereas the two non-invasive papillomas (stage pTa, TCCs 335 and 532) showed only 9p–, 9q22-q33–, and X–, and 7+, 9q–, and Y–, respectively. Both invasive tumors showed changes (1q22–24+, 2q14.1-qter–, 3q12-q13.3–, 6q12-q22–, 9q34+, 11q12-q13+, 17+, and 20q11.2-q12+) that are typical for their disease stage, as well as additional alterations, some of which are shown in Fig. 1. Areas with gains and losses deviated from the normal copy number to some extent, and the average numerical deviation from normal was 0.4-fold in the case of TCC 733 and 0.3-fold for TCC 827. The largest changes, amounting to at least a doubling of chromosomal content, were observed at 1q23 in TCC 733 (Fig. 1*A*) and 20q12 in TCC 827 (Fig. 1*B*).

mRNA Expression in Relation to DNA Copy Number—The mRNA levels from the two invasive tumors (TCCs 827 and 733) were compared with the two non-invasive counterparts (TCCs 532 and 335). This was done in two separate experiments in which we compared TCCs 733 to 335 and 827 to 532, respectively, using two different scaling settings for the arrays to rule out scaling as a confounding parameter. Approximately 1,800 genes that yielded a signal on the arrays were searched in the Unigene and Genemap data bases for chromosomal location, and those with a known location (1096) were plotted as bars covering their purported locus. In that way it was possible to construct a graphic presentation of DNA copy number and relative mRNA levels along the individual chromosomes (Fig. 1).

For each mRNA a ratio was calculated between the level in the invasive *versus* the non-invasive counterpart. Bars, which represent chromosomal location of a gene, were color-coded according to the expression ratio, and only differences larger than 2-fold were regarded as informative (Fig. 1). The density of genes along the chromosomes varied, and areas containing only one gene were excluded from the calculations. The resolution of the CGH method is very low, and some of the outlier data may be because of the fact that the boundaries of the chromosomal aberrations are not known at high resolution.

Two sets of calculations were made from the data. For the first set we used CGH alterations as the independent variable and estimated the frequency of expression alterations in these chromosomal areas. In general, areas with a strong gain of chromosomal material contained a cluster of genes having increased mRNA expression. For example, both chromosomes 1q21-q25, 2p and 9q, showed a relative gain of more than 100% in DNA copy number that was accompanied by increased mRNA expression levels in the two tumor pairs (Fig. 1). In most cases, chromosomal gains detected by CGH were accompanied by an increased level of transcripts in both TCCs 733 (77%) and 827 (80%) (Table I, *top*). Chromosomal losses, on the other hand, were not accompanied by decreased expression in several cases, and were often registered as having unaltered RNA levels (Table I, *top*). The inability to detect RNA expression changes in these cases was not because of fewer genes mapping to the lost regions (data not shown).

In the second set of calculations we selected expression alterations above 2-fold as the independent variable and estimated the frequency of CGH alterations in these areas. As above, we found that increased transcript expression correlated with gain of chromosomal material (TCC 733, 69% and TCC 827, 59%), whereas reduced expression was often detected in areas with unaltered CGH ratios (Table I, *bottom*). Furthermore, as a control we looked at areas with no alter-

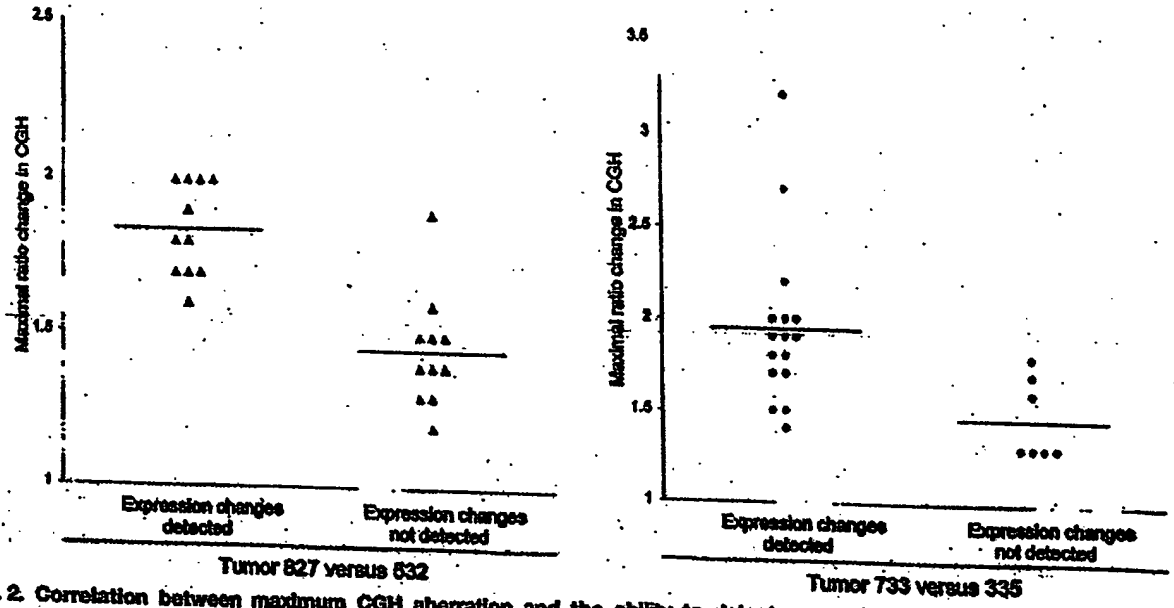


FIG. 2. Correlation between maximum CGH aberration and the ability to detect expression change by oligonucleotide array monitoring. The aberration is shown as a numerical -fold change in ratio between invasive tumors 827 (▲) and 733 (♦) and their non-invasive counterparts 532 and 335. The expression change was taken from the *Expression* line to the *right* in Fig. 1, which depicts the resulting expression change for a given chromosomal region. At least half of the mRNAs from a given region have to be either up- or down-regulated to be scored as an expression change. All chromosomal arms in which the CGH ratio plus or minus one standard deviation was outside the ratio value of one were included.

ation in expression. No alteration was detected by CGH in most of these areas (TCC 733, 60% and TCC 827, 81%; see Table I, *bottom*). Because the ability to observe reduced or increased mRNA expression clustering to a certain chromosomal area clearly reflected the extent of copy number changes, we plotted the maximum CGH aberrations in the regions showing CGH changes against the ability to detect a change in mRNA expression as monitored by the oligonucleotide arrays (Fig. 2). For both tumors TCC 733 ($p < 0.015$) and TCC 827 ($p < 0.00003$) a highly significant correlation was observed between the level of CGH ratio change (reflecting the DNA copy number) and alterations detected by the array based technology (Fig. 2). Similar data were obtained when areas with altered expression were used as independent variables. These areas correlated best with CGH when the CGH ratio deviated 1.6- to 2.0-fold (Table I, *bottom*) but mostly did not at lower CGH deviations. These data probably reflect that loss of an allele may only lead to a 50% reduction in expression level, which is at the cut-off point for detection of expression alterations. Gain of chromosomal material can occur to a much larger extent.

Microsatellite-based Detection of Minor Areas of Losses—In TCC 733, several chromosomal areas exhibiting DNA amplification were preceded or followed by areas with a normal CGH but reduced mRNA expression (see Fig. 1, TCC 733 chromosome 1q32, 2p21, and 7q21 and q32, 9q34, and 10q22). To determine whether these results were because of undetected loss of chromosomal material in these regions or because of other non-structural mechanisms regulating transcription, we examined two microsatellites positioned at chromosome 1q25–32 and two at chromosome 2p22. Loss of heterozygosity (LOH) was found at both 1q25 and at 2p22 indicating that minor deleted areas were not detected with the resolution of CGH (Fig. 3). Additionally, chromosome 2p in TCC 733 showed a CGH pattern of gain/no change/gain of DNA that correlated with transcript increase/decrease/increase. Thus, for the areas showing increased expression there was a correlation with the DNA copy number alterations (Fig. 1*A*). As indicated above, the mRNA decrease observed in the middle of the chromosomal gain was because of LOH, implying that one of the mechanisms for mRNA down-regulation may be regions that have undergone smaller losses of chromosomal material. However, this cannot be detected with the resolution of the CGH method.

In both TCC 733 and TCC 827, the telomeric end of chromosome 11p showed a normal ratio in the CGH analysis; however, clusters of five and three genes, respectively, lost their expression. Two microsatellites (D11S1760, D11S922) positioned close to MUC2, IGF2, and cathepsin D indicated LOH as the most likely mechanism behind the loss of expression (data not shown).

A reduced expression of mRNA observed in TCC 733 at chromosomes 3q24, 11p11, 12p12.2, 12q21.1, and 16q24 and in TCC 827 at chromosome 11p15.5, 12p11, 15q11.2, and 18q12 was also examined for chromosomal losses using microsatellites positioned as close as possible to the gene loci

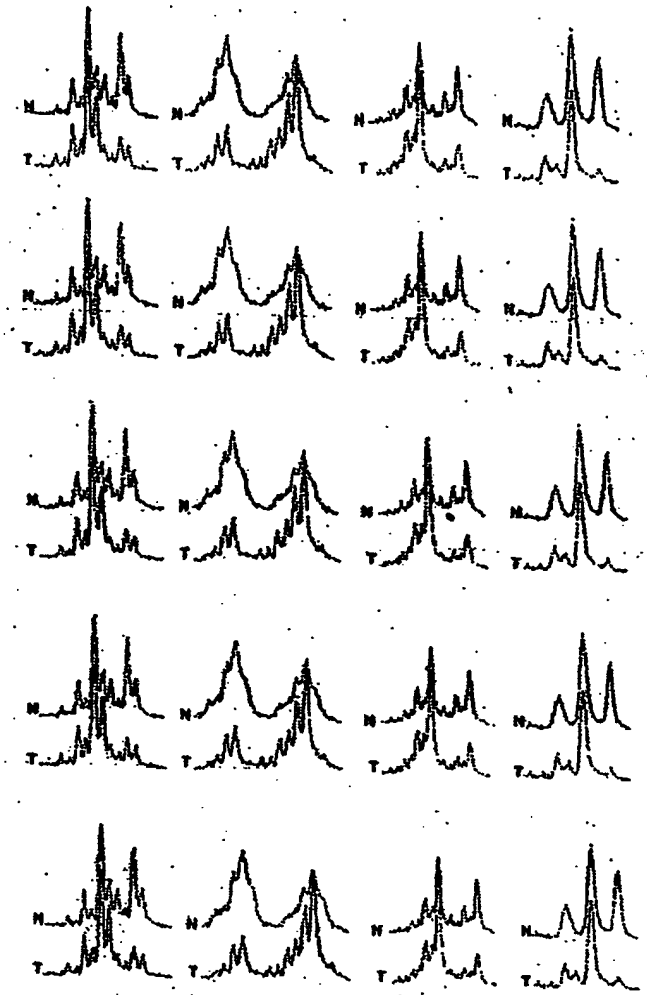


FIG. 3. Microsatellite analysis of loss of heterozygosity. Tumor 733 showing loss of heterozygosity at chromosome 1q25, detected (*a*) by D1S215 close to Hu class I histocompatibility antigen (gene number 38 in Fig. 1), (*b*) by D1S2735 close to cathepsin E (gene number 41 in Fig. 1), and (*c*) at chromosome 2p23 by D2S2251 close to general β-spectrin (gene number 11 on Fig. 1) and of (*d*) tumor 827 showing loss of heterozygosity at chromosome 18q12 by S18S1118 close to mitochondrial 3-oxoacyl-coenzyme A thiolase (gene number 12 in Fig. 1). The *upper curves* show the electropherogram obtained from normal DNA from leukocytes (*N*), and the *lower curves* show the electropherogram from tumor DNA (*T*). In all cases one allele is partially lost in the tumor amplicon.

showing reduced mRNA transcripts. Only the microsatellite positioned at 18q12 showed LOH (Fig. 3), suggesting that transcriptional down-regulation of genes in the other regions may be controlled by other mechanisms.

Relation between Changes in mRNA and Protein Levels—2D-PAGE analysis, in combination with Coomassie Brilliant Blue and/or silver staining, was carried out on all four tumors using fresh biopsy material. 40 well resolved abundant known proteins migrating in areas away from the edges of the pH

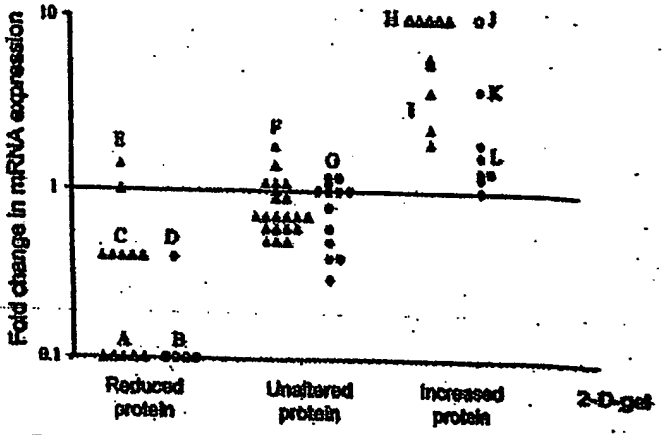


FIG. 4. Correlation between protein levels as judged by 2D-PAGE and transcript ratio. For comparison proteins were divided in three groups, unaltered in level or up- or down-regulated (*horizontal axis*). The mRNA ratio as determined by oligonucleotide arrays was plotted for each gene (*vertical axis*). ▲, mRNAs that were scored as present in both tumors used for the ratio calculation; Δ, mRNAs that were scored as absent in the invasive tumors (along *horizontal axis*) or as absent in non-invasive reference (*top* of figure). Two different scalings were used to exclude scaling as a confounder. TCCs 827 and 532 (▲Δ) were scaled with background suppression, and TCCs 733 and 335 (●○) were scaled without suppression. Both comparisons showed highly significant ($p < 0.005$) differences in mRNA ratios between the groups. Proteins shown were as follows: Group *A* (from *left*), phosphoglucomutase-1, glutathione transferase class-μ number 4, fatty acid-binding protein homologue, cytokeratin 18, and cytokeratin 13; *B* (from *left*), fatty acid-binding protein homologue, 28-kDa heat shock protein, cytokeratin 13, and calcyclin; *C* (from *left*), α-enolase, hnRNP B1, 28-kDa heat shock protein, 14-3-3-ε, and pre-mRNA splicing factor; *D*, mesothelial keratin K7 (type II); *E* (from *top*), glutathione *S*-transferase-π and mesothelial keratin K7 (type II); *F* (from *top* and *left*), adenylyl cyclase-associated protein, E-cadherin, keratin 19, calgizzarin, phosphoglycerate mutase, annexin IV, cytoskeletal γ-actin, hnRNP A1, integral membrane protein calnexin (IP90), hnRNP H, brain-type clathrin light chain-a, hnRNP F, 70-kDa heat shock protein, heterogeneous nuclear ribonucleoprotein A/B, translationally controlled tumor protein, liver glyceraldehyde-3-phosphate dehydrogenase, keratin 8, aldehyde reductase, and Na,K-ATPase β-1 subunit; *G*, (from *top* and *left*), TCP20, calgizzarin, 70-kDa heat shock protein, calnexin, hnRNP H, cytokeratin 15, ATP synthase, keratin 19, triosephosphate isomerase, hnRNP F, liver glyceraldehyde-3-phosphatase dehydrogenase, glutathione *S*-transferase-π, and keratin 8; *H* (from *left*), plasma gelsolin, autoantigen calreticulin, thioredoxin, and NAD+-dependent 15 hydroxyprostaglandin dehydrogenase; *I* (from *top*), prolyl 4-hydroxylase β-subunit, cytokeratin 20, cytokeratin 17, prohibition, and fructose 1,6-biphosphatase; *J* annexin II; *K*, annexin IV; *L* (from *top* and *left*), 90-kDa heat shock protein, prolyl 4-hydroxylase β-subunit, α-enolase, GRP 78, cyclophilin, and cofilin.

gradient, and having a known chromosomal location, were selected for analysis in the TCC pair 827/532. Proteins were identified by a combination of methods (see "Experimental Procedures"). In general there was a highly significant correlation ($p < 0.005$) between mRNA and protein alterations (Fig. 4). Only one gene showed disagreement between transcript alteration and protein alteration. Except for a group of cyto-

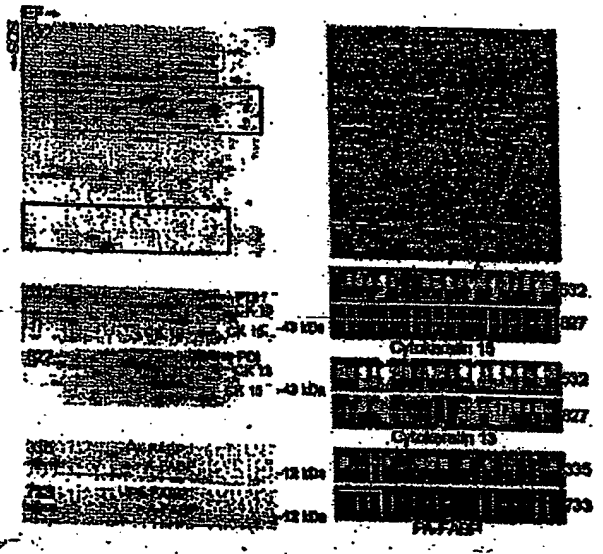


FIG. 5. Comparison of protein and transcript levels in invasive and non-invasive TCCs. The *upper part* of the figure shows a 2D gel (*left*) and the oligonucleotide array (*right*) of TCC 532. The *red rectangles* on the *upper gel* highlight the areas that are compared below. Identical areas of 2D gels of TCCs 532 and 827 are shown below. Clearly, cytokeratins 13 and 15 are strongly down-regulated in TCC 827 (*red annotation*). The tile on the array containing probes for cytokeratin 15 is enlarged *below* the array (*red arrow*) from TCC 532 and is compared with TCC 827. The *upper row* of squares in each tile corresponds to perfect match probes; the *lower row* corresponds to mismatch probes containing a mutation (used for correction for unspecific binding). Absence of signal is depicted as *black*, and the higher the signal the lighter the color. A high transcript level was detected in TCC 532 (6151 units) whereas a much lower level was detected in TCC 827 (absence of signals). For cytokeratin 13, a high transcript level was also present in TCC 532 (15659 units), and a much lower level was present in TCC 827 (623 units). The 2D gels at the *bottom* of the figure (*left*) show levels of PA-FABP and adipocyte-FABP in TCCs 335 and 733 (invasive), respectively. Both proteins are down-regulated in the invasive tumor. To the *right* we show the array tiles for the PA-FABP transcript. A medium transcript level was detected in the case of TCC 335 (1277 units) whereas very low levels were detected in TCC 733 (166 units). *IEF*, isoelectric focusing.

keratins encoded by genes on chromosome 17 (Fig. 5) the analyzed proteins did not belong to a particular family. 28 well focused proteins whose genes had a know chromosomal location were detected in TCCs 733 and 335, and of these 19 correlated ($p < 0.005$) with the mRNA changes detected using the arrays (Fig. 4). For example, PA-FABP was highly expressed in the non-invasive TCC 335 but lost in the invasive counterpart (TCC 733; see Fig. 5). The smaller number of proteins detected in both 733 and 335 was because of the smaller size of the biopsies that were available.

11 chromosomal regions where CGH showed aberrations that corresponded to the changes in transcript levels also showed corresponding changes in the protein level (Table II). These regions included genes that encode proteins that are found to be frequently altered in bladder cancer, namely cytokeratins 17 and 20, annexins II and IV, and the fatty acid-binding proteins PA-FABP and FBP1. Four of these proteins were encoded by genes in chromosome 17q, a frequently amplified chromosomal area in invasive bladder cancers.

DISCUSSION

Most human cancers have abnormal DNA content, having lost some chromosomal parts and gained others. The present study provides some evidence as to the effect of these gains and losses on gene expression in two pairs of non-invasive and invasive TCCs using high throughput expression arrays and proteomics, in combination with CGH. In general, the results showed that there is a clear individual regulation of the mRNA expression of single genes, which in some cases was superimposed by a DNA copy number effect. In most cases, genes located in chromosomal areas with gains often exhibited increased mRNA expression, whereas areas showing losses showed either no change or a reduced mRNA expression. The latter might be because of the fact that losses most often are restricted to loss of one allele, and the cut-off point for detection of expression alterations was a 2-fold change, thus being at the border of detection. In several cases, how-

TABLE II

Proteins whose expression level correlates with both mRNA and gene dose changes

Protein	Chromosomal location	Tumor TCC	CGH alteration	Transcript alteration[a]	Protein alteration
Annexin II	1q21	733	Gain	Abs to Pres[a]	Increase
Annexin IV	2p13	733	Gain	3.9-Fold up	Increase
Cytokeratin 17	17q12-q21	827	Gain	3.8-Fold up	Increase
Cytokeratin 20	17q21.1	827	Gain	5.6-Fold up	Increase
(PA-)FABP	8q21.2	827	Loss	10-Fold down	Decrease
FBP1	9q22	827	Gain	2.3-Fold up	Increase
Plasma gelsolin	9q31	827	Gain	Abs to Pres	Increase
Heat shock protein 28	15q12-q13	827	Loss	2.5-Fold up	Decrease
Prohibitin	17q21	827/733	Gain	3.7-/2.5-Fold up[b]	Increase
Prolyl-4-hydroxyl	17q25	827/733	Gain	5.7-/1.6-Fold up	Increase
hnRNPB1	7p15	827	Loss	2.5-Fold down	Decrease

[a] Abs, absent; Pres, present.

[b] In cases where the corresponding alterations were found in both TCCs 827 and 733 these are shown as 827/733.

ever, an increase or decrease in DNA copy number was associated with *de novo* occurrence or complete loss of transcript, respectively. Some of these transcripts could not be detected in the non-invasive tumor but were present at relatively high levels in areas with DNA amplifications in the invasive tumors (e.g. in TCC 733 transcript from cellular ligand of annexin II gene (chromosome 1q21) from absent to 2670 arbitrary units; in TCC 827 transcript from small proline-rich protein 1 gene (chromosome 1q12-q21.1) from absent to 1326 arbitrary units). It may be anticipated from these data that significant clustering of genes with an increased expression to a certain chromosomal area indicates an increased likelihood of gain of chromosomal material in this area.

Considering the many possible regulatory mechanisms acting at the level of transcription, it seems striking that the gene dose effects were so clearly detectable in gained areas. One hypothetical explanation may lie in the loss of controlled methylation in tumor cells (17–19). Thus, it may be possible that in chromosomes with increased DNA copy numbers two or more alleles could be demethylated simultaneously leading to a higher transcription level, whereas in chromosomes with losses the remaining allele could be partly methylated, turning off the process (20, 21). A recent report has documented a ploidy regulation of gene expression in yeast, but in this case all the genes were present in the same ratio (22), a situation that is not analogous to that of cancer cells, which show marked chromosomal aberrations, as well as gene dosage effects.

Several CGH studies of bladder cancer have shown that some chromosomal aberrations are common at certain stages of disease progression, often occurring in more than 1 of 3 tumors. In pTa tumors, these include 9p−, 9q−, 1q+, Y− (2, 6), and in pT1 tumors, 2q−, 11p−, 11q−, 1q+, 5p+, 8q+, 17q+, and 20q+ (2–4, 6, 7). The pTa tumors studied here showed similar aberrations such as 9p− and 9q22-q33− and 9q− and Y−, respectively. Likewise, the two minimal invasive pT1 tumors showed aberrations that are commonly seen at that stage, and TCC 827 had a remarkable resemblance to the commonly seen pattern of losses and gains, such as 1q22–24 amplification (seen in both tumors); 11q14-q22 loss, the latter often linked to 17 q+ (both tumors), and 1q+ and 9p−, often linked to 20q+ and 11 q13+ (both tumors) (7–9). These observations indicate that the pairs of tumors used in this study exhibit chromosomal changes observed in many tumors, and therefore the findings could be of general importance for bladder cancer.

Considering that the mapping resolution of CGH is of about 20 megabases it is only possible to get a crude picture of chromosomal instability using this technique. Occasionally, we observed reduced transcript levels close to or inside regions with increased copy numbers. Analysis of these regions by positioning heterozygous microsatellites as close as possible to the locus showing reduced gene expression revealed loss of heterozygosity in several cases. It seems likely that multiple and different events occur along each chromosomal arm and that the use of cDNA microarrays for analysis of DNA copy number changes will reach a resolution that can resolve these changes, as has recently been proposed (2). The outlier data were not more frequent at the boundaries of the CGH aberrations. At present we do not know the mechanism behind chromosomal aneuploidy and cannot predict whether chromosomal gains will be transcribed to a larger extent than the two native alleles. A mechanism as genetic imprinting has an impact on the expression level in normal cells and is often reduced in tumors. However, the relation between imprinting and gain of chromosomal material is not known.

We regard it as a strength of this investigation that we were able to compare invasive tumors to benign tumors rather than to normal urothelium, as the tumors studied were biologically very close and probably may represent successive steps in the progression of bladder cancer. Despite the limited amount of fresh tissue available it was possible to apply three different state of the art methods. The observed correlation between DNA copy number and mRNA expression is remarkable when one considers that different pieces of the tumor biopsies were used for the different sets of experiments. This indicate that bladder tumors are relatively homogenous, a notion recently supported by CGH and LOH data that showed a remarkable similarity even between tumors and distant metastasis (10, 23).

In the few cases analyzed, mRNA and protein levels showed a striking correspondence although in some cases we found discrepancies that may be attributed to translational regulation, post-translational processing, protein degradation, or a combination of these. Some transcripts belong to undertranslated mRNA pools, which are associated with few translationally inactive ribosomes; these pools, however, seem to be rare (24). Protein degradation, for example, may be very important in the case of polypeptides with a short half-life (e.g. signaling proteins). A poor correlation between mRNA and protein levels was found in liver cells as determined by arrays and 2D-PAGE (25), and a moderate correlation was recently reported by Ideker *et al.* (26) in yeast.

Interestingly, our study revealed a much better correlation between gained chromosomal areas and increased mRNA levels than between loss of chromosomal areas and reduced mRNA levels. In general, the level of CGH change determined the ability to detect a change in transcript. One possible explanation could be that by losing one allele the change in mRNA level is not so dramatic as compared with gain of material, which can be rather unlimited and may lead to a severalfold increase in gene copy number resulting in a much higher impact on transcript level. The latter would be much easier to detect on the expression arrays as the cut-off point was placed at a 2-fold level so as not to be biased by noise on the array. Construction of arrays with a better signal to noise ratio may in the future allow detection of lesser than 2-fold alterations in transcript levels, a feature that may facilitate the analysis of the effect of loss of chromosomal areas on transcript levels.

In eleven cases we found a significant correlation between DNA copy number, mRNA expression, and protein level. Four of these proteins were encoded by genes located at a frequently amplified area in chromosome 17q. Whether DNA copy number is one of the mechanisms behind alteration of these eleven proteins is at present unknown and will have to be proved by other methods using a larger number of samples. One factor making such studies complicated is the large extent of protein modification that occurs after translation, requiring immunoidentification and/or mass spectrometry to correctly identify the proteins in the gels.

In conclusion, the results presented in this study exemplify the large body of knowledge that may be possible to gather in the future by combining state of the art techniques that follow the pathway from DNA to protein (26). Here, we used a traditional chromosomal CGH method, but in the future high resolution CGH based on microarrays with many thousand radiation hybrid-mapped genes will increase the resolution and information derived from these types of experiments (2). Combined with expression arrays analyzing transcripts derived from genes with known locations, and 2D gel analysis to obtain information at the post-translational level, a clearer and more developed understanding of the tumor genome will be forthcoming.

Acknowledgments—We thank Mie Madsen, Hanne Steen, Inge Lis Thorsen, Hans Lund, Vikolaj Ørntoft, and Lynn Bjerke for technical help and Thomas Gingeras, Christine Harrington, and Morten Østergaard for valuable discussions.

* This work was supported by grants from The Danish Cancer Society, the University of Aarhus, Aarhus County, Novo Nordic, the Danish Biotechnology Program, the Frenkels Foundation, the John and Birthe Meyer Foundation, and NCI, National Institutes of Health Grant CA47537. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ To whom correspondence should be addressed: Dept. of Clinical Biochemistry, Molecular Diagnostic Laboratory, Aarhus University Hospital, Skejby, DK-8200 Aarhus N, Denmark. Tel.: 45-89495100/45-86156201 (private); Fax: 45-89496018; E-mail: ornloft@klba.sks.au.dk.

REFERENCES

1. Lengauer, C., Kinzler, K. W., and Vogelstein, B. (1998) Genetic instabilities in human cancers. *Nature* 17, 643–649
2. Pollack, J. R., Perou, C. M., Alizadeh, A. A., Eisen, M. B., Pergamenschikov, A., Williams, C. F., Jeffrey, S. S., Botstein, D., and Brown, P. O. (1999) Genome-wide analysis of DNA copy-number changes using cDNA microarrays. *Nat. Genet.* 23, 41–46
3. de Cremoux, P., Martin, E. C., Vincent-Salomon, A., Dieras, V., Barbaroux, C., Liva, S., Poullart, P., Sastre-Garau, X., and Magdelenat, H. (1999) Quantitative PCR analysis of c-erb B-2 (HER2/neu) gene amplification and comparison with p185(HER2/neu) protein expression in breast cancer drill biopsies. *Int. J. Cancer* 83, 157–161
4. Brunger, P. P., Tamimi, Y., Shuuring, E., and Schalken, J. (1996) Expression of cyclin D1 and EMS1 in bladder tumors; relationship with chromosome 11q13 amplifications. *Oncogene* 12, 1747–1753
5. Slavc, I., Ellenbogen, R., Jung, W. H., Vawter, G. F., Kretschmar, C., Grier, H., and Kort, B. R. (1990) *myc* gene amplification and expression in primary human neuroblastoma. *Cancer Res.* 50, 1459–1463
6. Sauter, G., Carroll, P., Moch, H., Kallioniemi, A., Kerschmann, R., Narayan, P., Mihatsch, M. J., and Waldman, F. M. (1995) *c-myc* copy-number gains in bladder cancer detected by fluorescence *in situ* hybridization. *Am J. Pathol.* 146, 1131–1139
7. Richter, J., Jiang, F., Gorog, J. P., Sartorious, G., Egenter, C., Gasser, T. C., Moch, H., Mihatsch, M. J., and Sauter, G. (1997) Marked genetic differences between stage pTa and stage pT1 papillary bladder cancer detected by comparative genomic hybridization. *Cancer Res.* 57, 2860–2864
8. Richter, J., Beffa, L., Wagner, U., Schraml, P., Gasser, T. C., Moch, H., Mihatsch, M. J., and Sauter, G. (1998) Patterns of chromosomal imbalances in advanced urinary bladder cancer detected by comparative genomic hybridization. *Am. J. Pathol.* 153, 1615–1621
9. Bruch, J., Wohr, G., Hautmann, R., Mattfeldt, T., Bruderlein, S., Moller, P., Sauter, S., Hameister, H., Vogel, W., and Paiss, T. (1998) Chromosomal changes during progression of transitional cell carcinoma of the bladder and delineation of the amplified interval on chromosome arm 8q. *Genes Chromosomes Cancer* 23, 167–174
10. Hovey, R. M., Chu, L., Balaza, M., De Vries, S., Moore, D., Sauter, G., Carroll, P. R., and Waldman, F. M. (1998) Genetic alterations in primary bladder cancers and their metastases. *Cancer Res.* 15, 5555–5560
11. Simon, R., Burger, H., Brinkschmidt, C., Bocker, W., Hertle, L., and Terpe, H. J. (1998) Chromosomal aberrations associated with invasion in papillary superficial bladder cancer. *J. Pathol.* 186, 345–351
12. Koo, S. H., Kwon, K. C., Ihm, C. R., Jeon, Y. M., Park, J. W., and Suh, C. K. (1999) Detection of genetic alterations in bladder tumors by comparative genomic hybridization and cytogenetic analysis. *Cancer Genet. Cytogenet.* 110, 87–93
13. Wodicka, L., Dong, H., Mittmann, M., Ho, M. H., and Lockhart, D. J. (1997) Genome-wide expression monitoring in *Saccharomyces cerevisiae*. *Nat. Biotechnol.* 15, 1359–1367
14. Christensen, M., Sunde, L., Bolund, L., and Ornloft, T. F. (1999) Comparison of three methods of microsatellite detection. *Scand. J. Clin. Lab. Invest.* 59, 167–177
15. Celis, J. E., Ostergaard, M., Basse, B., Celis, A., Lauridsen, J. B., Ratz, G. P., Andersen, I., Hein, B., Wolf, H., Ornloft, T. F., and Rasmussen, H. H. (1996) Loss of adipocyte-type fatty acid binding protein and other protein biomarkers is associated with progression of human bladder transitional cell carcinomas. *Cancer Res.* 56, 4782–4790
16. Celis, J. E., Ratz, G., Basse, B., Lauridsen, J. B., and Celis, A. (1994) In *Cell Biology: A Laboratory Handbook* (Celis, J. E., ed) Vol. 3, pp. 222–230, Academic Press, Orlando, FL
17. Ohlsson, R., Tycko, B., and Sapienza, C. (1996) Monoallelic expression: 'there can only be one'. *Trends Genet.* 14, 435–438
18. Hollander, G. A., Zuklys, S., Morel, C., Mizoguchi, E., Mobisson, K., Simpson, S., Terhorst, C., Wishart, W., Golan, D. E., Bhan, A. K., and Buraikoff, S. J. (1998) Monoallelic expression of the interleukin-2 locus. *Science* 279, 2118–2121
19. Brannan, C. I., and Bartolomei, M. S. (1999) Mechanisms of genomic imprinting. *Curr. Opin. Genet. Dev.* 9, 164–170
20. Ohlsson, R., Cui, H., He, L., Pfeifer, S., Malmikumpa, H., Jiang, S., Feinberg, A. P., and Hedborg, F. (1999) Mosaic allelic insulin-like growth factor 2 expression patterns reveal a link between Wilms' tumorigenesis and epigenetic heterogeneity. *Cancer Res.* 59, 3899–3902
21. Cui, H., Hedborg, F., He, L., Nordenskjold, A., Sandstedt, B., Pfeifer-Ohlsson, S., and Ohlsson, R. (1997) Inactivation of H19, an imprinted and putative tumor repressor gene, is a preneoplastic event during Wilms' tumorigenesis. *Cancer Res.* 57, 4469–4473
22. Gelitski, T., Saldanha, A. J., Styles, C. A., Lander, E. S., and Fink, G. R. (1999) Ploidy regulation of gene expression. *Science* 285, 251–254
23. Tsao, J., Yatabe, Y., Marki, I. D., Hajyan, K., Jones, P. A., and Shibata, D. (2000) Bladder cancer genotype stability during clinical progression. *Genes Chromosomes Cancer* 29, 26–32
24. Zong, Q., Schummer, M., Hood, L., and Morris, D. R. (1999) Messenger RNA translation state: the second dimension of high-throughput expression screening. *Proc. Natl. Acad. Sci. U. S. A.* 96, 10632–10636
25. Anderson, L., and Seilhamer, J. (1997) Comparison of selected mRNA and protein abundances in human liver. *Electrophoresis* 18, 533–537
26. Ideker, T., Thorsson, V., Ranish, J. A., Christmas, R., Buhler, J., Eng, J. K., Bumgarner, R., Goodlett, D. R., Aebersold, R., and Hood, L. (2001) Integrated genomic and proteomic analyses of a systematically perturbed metabolic network. *Science* 292, 929–934

[CANCER RESEARCH 62, 6240–6245, November 1, 2002]

Impact of DNA Amplification on Gene Expression Patterns in Breast Cancer[1,2]

Elizabeth Hyman,[3] Päivikki Kauraniemi,[3] Sampsa Hautaniemi, Maija Wolf, Spyro Mousses, Ester Rozenblum, Markus Ringnér, Guido Sauter, Outi Monni, Abdel Elkahloun, Olli-P. Kallioniemi, and Anne Kallioniemi[4]

Howard Hughes Medical Institute-NIH Research Scholar, Bethesda, Maryland 20892 [E. H.]; Cancer Genetics Branch, National Human Genome Research Institute, NIH, Bethesda, Maryland 20892 [E. H., P. K., S. H., M. W., S. M., E. R., M. R., A. E., O. K., A. K.]; Laboratory of Cancer Genetics, Institute of Medical Technology, University of Tampere and Tampere University Hospital, FIN-33520 Tampere, Finland [P. K., A. K.]; Signal Processing Laboratory, Tampere University of Technology, FIN-33101 Tampere, Finland [S. H.]; Institute of Pathology, University of Basel, CH-4003 Basel, Switzerland [G. S.]; and Biomedicum Biochip Center, Helsinki University Hospital, Biomedicum Helsinki, FIN-00014 Helsinki, Finland [O. M.]

ABSTRACT

Genetic changes underlie tumor progression and may lead to cancer-specific expression of critical genes. Over 1100 publications have described the use of comparative genomic hybridization (CGH) to analyze the pattern of copy number alterations in cancer, but very few of the genes affected are known. Here, we performed high-resolution CGH analysis on cDNA microarrays in breast cancer and directly compared copy number and mRNA expression levels of 13,824 genes to quantitate the impact of genomic changes on gene expression. We identified and mapped the boundaries of 24 independent amplicons, ranging in size from 0.2 to 12 Mb. Throughout the genome, both high- and low-level copy number changes had a substantial impact on gene expression, with 44% of the highly amplified genes showing overexpression and 10.5% of the highly overexpressed genes being amplified. Statistical analysis with random permutation tests identified 270 genes whose expression levels across 14 samples were systematically attributable to gene amplification. These included most previously described amplified genes in breast cancer and many novel targets for genomic alterations, including the *HOXB7* gene, the presence of which in a novel amplicon at 17q21.3 was validated in 10.2% of primary breast cancers and associated with poor patient prognosis. In conclusion, CGH on cDNA microarrays revealed hundreds of novel genes whose overexpression is attributable to gene amplification. These genes may provide insights to the clonal evolution and progression of breast cancer and highlight promising therapeutic targets.

INTRODUCTION

Gene expression patterns revealed by cDNA microarrays have facilitated classification of cancers into biologically distinct categories, some of which may explain the clinical behavior of the tumors (1–6). Despite this progress in diagnostic classification, the molecular mechanisms underlying gene expression patterns in cancer have remained elusive, and the utility of gene expression profiling in the identification of specific therapeutic targets remains limited.

Accumulation of genetic defects is thought to underlie the clonal evolution of cancer. Identification of the genes that mediate the effects of genetic changes may be important by highlighting transcripts that are actively involved in tumor progression. Such transcripts and their encoded proteins would be ideal targets for anticancer therapies, as demonstrated by the clinical success of new therapies against amplified oncogenes, such as *ERBB2* and *EGFR* (7, 8), in breast cancer and other solid tumors. Besides amplifications of known oncogenes, over 20 recurrent regions of DNA amplification have been mapped in breast cancer by CGH[5] (9, 10). However, these amplicons are often large and poorly defined, and their impact on gene expression remains unknown.

We hypothesized that genome-wide identification of those gene expression changes that are attributable to underlying gene copy number alterations would highlight transcripts that are actively involved in the causation or maintenance of the malignant phenotype. To identify such transcripts, we applied a combination of cDNA and CGH microarrays to: (*a*) determine the global impact that gene copy number variation plays in breast cancer development and progression; and (*b*) identify and characterize those genes whose mRNA expres-

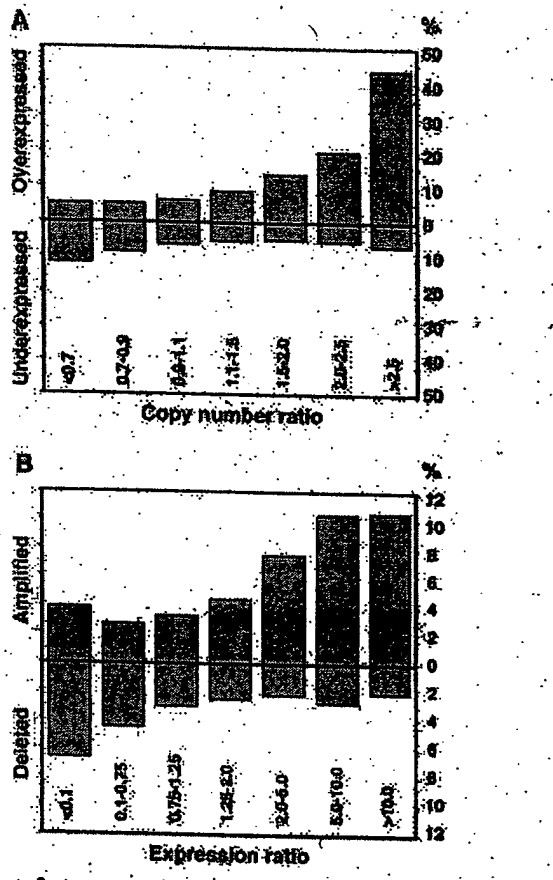


Fig. 1. Impact of gene copy number on global gene expression levels. *A*, percentage of over- and underexpressed genes (*Y axis*) according to copy number ratios (*X axis*). Threshold values used for over- and underexpression were >2.184 (global upper 7% of the cDNA ratios) and <0.4826 (global lower 7% of the expression ratios). *B*, percentage of amplified and deleted genes according to expression ratios. Threshold values for amplification and deletion were >1.5 and <0.7.

Received 5/29/02; accepted 8/28/02.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Supported in part by the Academy of Finland, Emil Aaltonen Foundation, the Finnish Cancer Society, the Pirkanmaa Cancer Society, the Pirkanmaa Cultural Foundation, the Finnish Breast Cancer Group, the Foundation for the Development of Laboratory Medicine, the Medical Research Fund of the Tampere University Hospital, the Foundation for Commercial and Technical Sciences, and the Swedish Research Council.

[2] Supplementary data for this article are available at Cancer Research Online (http://cancerres.aacrjournals.org).

[3] Contributed equally to this work.

[4] To whom requests for reprints should be addressed, at Laboratory of Cancer Genetics, Institute of Medical Technology, Lenkkeilijankatu 6, FIN-33520 Tampere, Finland. Phone: 358-3247-4125; Fax: 358-3247-4168; E-mail: anne.kallioniemi@uta.fi.

[5] The abbreviations used are: CGH, comparative genomic hybridization; FISH, fluorescence *in situ* hybridization; RT-PCR, reverse transcription-PCR.

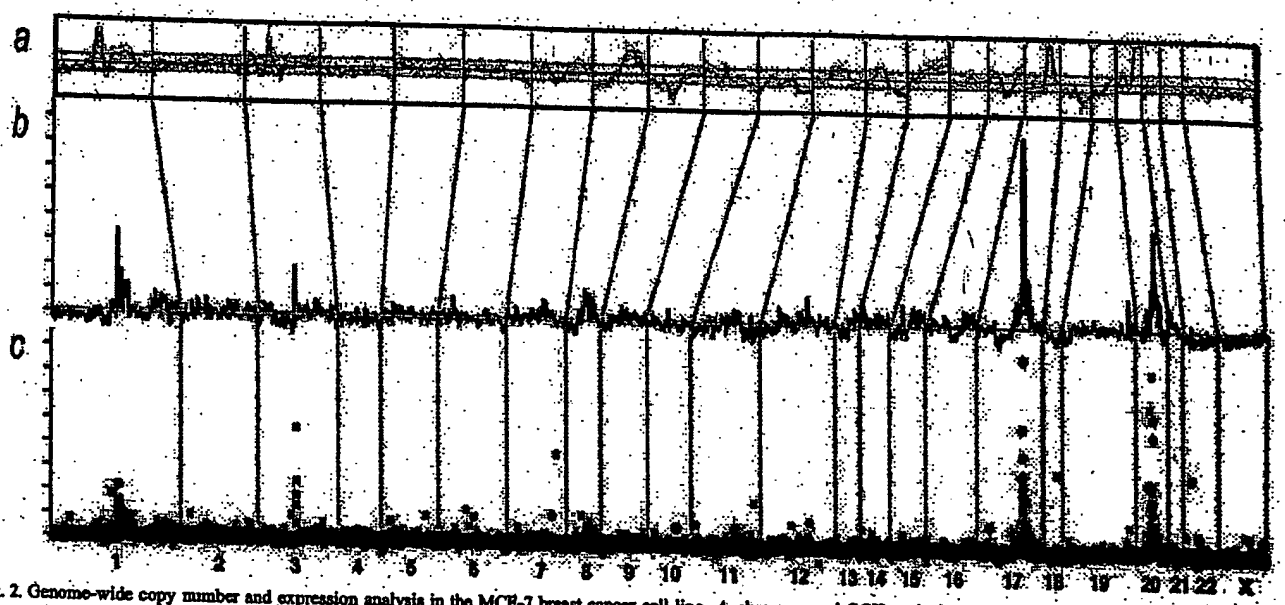


Fig. 2. Genome-wide copy number and expression analysis in the MCF-7 breast cancer cell line. *A*, chromosomal CGH analysis of MCF-7. The copy number ratio profile (*blue line*) across the entire genome from 1p telomere to Xq telomere is shown along with ±1 SD (*orange lines*). The *black horizontal line* indicates a ratio of 1.0; *red line*, a ratio of 0.8; and *green line*, a ratio of 1.2. *B–C*, genome-wide copy number analysis in MCF-7 by CGH on cDNA microarray. The copy number ratios were plotted as a function of the position of the cDNA clones along the human genome. In *B*, individual data points are connected with a line, and a moving median of 10 adjacent clones is shown. *Red horizontal line*, the copy number ratio of 1.0. In *C*, individual data points are labeled by color coding according to cDNA expression ratios. The *bright red dots* indicate the upper 2%, and *dark red dots*, the next 5% of the expression ratios in MCF-7 cells (overexpressed genes); *bright green dots* indicate the lowest 2%, and *dark green dots*, the next 5% of the expression ratios (underexpressed genes); the rest of the observations are shown with *black crosses*. The chromosome numbers are shown at the *bottom* of the figure, and chromosome boundaries are indicated with a *dashed line*.

sion is most significantly associated with amplification of the corresponding genomic template.

MATERIALS AND METHODS

Breast Cancer Cell Lines. Fourteen breast cancer cell lines (BT-20, BT-474, HCC1428, Hs578t, MCF7, MDA-361, MDA-436, MDA-453, MDA-468, SKBR-3, T-47D, UACC812, ZR-75-1, and ZR-75-30) were obtained from the American Type Culture Collection (Manassas, VA). Cells were grown under recommended culture conditions. Genomic DNA and mRNA were isolated using standard protocols.

Copy Number and Expression Analyses by cDNA Microarrays. The preparation and printing of the 13,824 cDNA clones on glass slides were performed as described (11–13). Of these clones, 244 represented uncharacterized expressed sequence tags, and the remainder corresponded to known genes. CGH experiments on cDNA microarrays were done as described (14, 15). Briefly, 20 μg of genomic DNA from breast cancer cell lines and normal human WBCs were digested for 14–18 h with *Alu*I and *Rsa*I (Life Technologies, Inc., Rockville, MD) and purified by phenol/chloroform extraction. Six μg of digested cell line DNAs were labeled with Cy3-dUTP (Amersham Pharmacia) and normal DNA with Cy5-dUTP (Amersham Pharmacia) using the Bioprime Labeling kit (Life Technologies, Inc.). Hybridization (14, 15) and posthybridization washes (13) were done as described. For the expression analyses, a standard reference (Universal Human Reference RNA; Stratagene, La Jolla, CA) was used in all experiments. Forty μg of reference RNA were labeled with Cy3-dUTP and 3.5 μg of test mRNA with Cy5-dUTP, and the labeled cDNAs were hybridized on microarrays as described (13, 15). For both microarray analyses, a laser confocal scanner (Agilent Technologies, Palo Alto, CA) was used to measure the fluorescence intensities at the target locations using the DEARRAY software (16). After background subtraction, average intensities at each clone in the test hybridization were divided by the average intensity of the corresponding clone in the control hybridization. For the copy number analysis, the ratios were normalized on the basis of the distribution of ratios of all targets on the array and for the expression analysis on the basis of 88 housekeeping genes, which were spotted four times onto the array. Low quality measurements (*i.e.*, copy number data with mean reference intensity <100 fluorescent units, and expression data with both test and reference intensity <100 fluorescent units and/or with spot size <50 units) were excluded from the analysis and were treated as missing values. The distributions of fluorescence ratios were used to define cutpoints for increased/decreased copy number. Genes with CGH ratio >1.43 (representing the upper 5% of the CGH ratios across all experiments) were considered to be amplified, and genes with ratio <0.73 (representing the lower 5%) were considered to be deleted.

Statistical Analysis of CGH and cDNA Microarray Data. To evaluate the influence of copy number alterations on gene expression, we applied the following statistical approach. CGH and cDNA calibrated intensity ratios were log-transformed and normalized using median centering of the values in each cell line. Furthermore, cDNA ratios for each gene across all 14 cell lines were median centered. For each gene, the CGH data were represented by a vector that was labeled 1 for amplification (ratio, >1.43) and 0 for no amplification. Amplification was correlated with gene expression using the signal-to-noise statistics (1). We calculated a weight, w_g, for each gene as follows:

$$w_g = \frac{m_{g1} - m_{g0}}{\sigma_{g1} + \sigma_{g0}}$$

where m_{g1}, σ_{g1} and m_{g0}, σ_{g0} denote the means and SDs for the expression levels for amplified and nonamplified cell lines, respectively. To assess the statistical significance of each weight, we performed 10,000 random permutations of the label vector. The probability that a gene had a larger or equal weight by random permutation than the original weight was denoted by α. A low α (<0.05) indicates a strong association between gene expression and amplification.

Genomic Localization of cDNA Clones and Amplicon Mapping. Each cDNA clone on the microarray was assigned to a Unigene cluster using the Unigene Build 141.[6] A database of genomic sequence alignment information for mRNA sequences was created from the August 2001 freeze of the University of California Santa Cruz's GoldenPath database.[7] The chromosome and bp positions for each cDNA clone were then retrieved by relating these data sets. Amplicons were defined as a CGH copy number ratio >2.0 in at least two adjacent clones in two or more cell lines or a CGH ratio >2.0 in at least three adjacent clones in a single cell line. The amplicon start and end positions were

[6] Internet address: http://research.nhgri.nih.gov/microarray/downloadable_cdna.html.
[7] Internet address: www.genome.ucsc.edu.

Table 1 *Summary of independent amplicons in 14 breast cancer cell lines by CGH microarray*

Location	Start (Mb)	End (Mb)	Size (Mb)
1p13	132.79	132.94	0.2
1q21	173.92	177.25	3.3
1q22	179.28	179.57	0.3
3p14	71.94	74.66	2.7
7p12.1–7p11.2	55.62	60.95	5.3
7q31	125.73	130.96	5.2
7q32	140.01	140.68	0.7
8q21.11–8q21.13	86.45	92.46	6.0
8q21.3	98.45	103.05	4.6
8q23.3–8q24.14	129.88	142.15	12.3
8q24.22	151.21	152.16	1.0
9p13	38.65	39.25	0.6
13q22–q31	77.15	81.38	4.2
16q22	86.70	87.62	0.9
17q11	29.30	30.85	1.6
17q12–q21.2	39.79	42.80	3.0
17q21.32–q21.33	52.47	55.80	3.3
17q22–q23.3	63.81	69.70	5.9
17q23.3–q24.3	69.93	74.99	5.1
19q13	40.63	41.40	0.8
20q11.22	34.59	35.85	1.3
20q13.12	44.00	45.62	1.6
20q13.12–q13.13	46.45	49.43	3.0
20q13.2–q13.32	51.32	59.12	7.8

extended to include neighboring nonamplified clones (ratio, <1.5). The amplicon size determination was partially dependent on local clone density.

FISH. Dual-color interphase FISH to breast cancer cell lines was done as described (17). Bacterial artificial chromosome clone RP11-361K8 was labeled with SpectrumOrange (Vysis, Downers Grove, IL), and SpectrumOrange-labeled probe for *EGFR* was obtained from Vysis. SpectrumGreen-labeled chromosome 7 and 17 centromere probes (Vysis) were used as a reference. A tissue microarray containing 612 formalin-fixed, paraffin-embedded primary breast cancers (17) was applied in FISH analyses as described (18). The use of these specimens was approved by the Ethics Committee of the University of Basel and by the NIH. Specimens containing a 2-fold or higher increase in the number of test probe signals, as compared with corresponding centromere signals, in at least 10% of the tumor cells were considered to be amplified. Survival analysis was performed using the Kaplan-Meier method and the log-rank test.

RT-PCR. The *HOXB7* expression level was determined relative to *GAPDH*. Reverse transcription and PCR amplification were performed using Access RT-PCR System (Promega Corp., Madison, WI) with 10 ng of mRNA as a template. *HOXB7* primers were 5′-GAGCAGAGGGACTCGGACTT-3′ and 5′-GCGTCAGGTAGCGATTGTAG-3′.

RESULTS

Global Effect of Copy Number on Gene Expression. 13,824 arrayed cDNA clones were applied for analysis of gene expression and gene copy number (CGH microarrays) in 14 breast cancer cell lines. The results illustrate a considerable influence of copy number on gene expression patterns. Up to 44% of the highly amplified transcripts (CGH ratio, >2.5) were overexpressed (*i.e.*, belonged to the global upper 7% of expression ratios), compared with only 6% for genes with normal copy number levels (Fig. 1A). Conversely, 10.5% of the transcripts with high-level expression (cDNA ratio, >10) showed increased copy number (Fig. 1*B*). Low-level copy number increases and decreases were also associated with similar, although less dramatic, outcomes on gene expression (Fig. 1).

Identification of Distinct Breast Cancer Amplicons. Base-pair locations obtained for 11,994 cDNAs (86.8%) were used to plot copy number changes as a function of genomic position (Fig. 2, Supplement Fig. A). The average spacing of clones throughout the genome was 267 kb. This high-resolution mapping identified 24 independent breast cancer amplicons, spanning from 0.2 to 12 Mb of DNA (Table 1). Several amplification sites detected previously by chromosomal CGH were validated, with 1q21, 17q12–q21.2, 17q22–q23, 20q13.1, and 20q13.2 regions being most commonly amplified. Furthermore, the boundaries of these amplicons were precisely delineated. In addition, novel amplicons were identified at 9p13 (38.65–39.25 Mb), and 17q21.3 (52.47–55.80 Mb).

Direct Identification of Putative Amplification Target Genes. The cDNA/CGH microarray technique enables the direct correlation of copy number and expression data on a gene-by-gene basis throughout the genome. We directly annotated high-resolution CGH plots with gene expression data using color coding. Fig. 2*C* shows that most of the amplified genes in the MCF-7 breast cancer cell line at 1p13, 17q22–q23, and 20q13 were highly overexpressed. A view of chromosome 7 in the MDA-468 cell line implicates *EGFR* as the most highly overexpressed and amplified gene at 7p11–p12 (Fig. 3*A*). In BT-474, the two known amplicons at 17q12 and 17q22–q23 contained numerous highly overexpressed genes (Fig. 3*B*). In addition, several genes, including the homeobox genes *HOXB2* and *HOXB7*, were highly amplified in a previously undescribed independent amplicon at 17q21.3. *HOXB7* was systematically amplified (as validated by FISH, Fig. 3*B*, *inset*) as well as overexpressed (as verified by RT-PCR, data not shown) in BT-474, UACC812, and ZR-75-30 cells. Furthermore, this novel

Fig. 3. Annotation of gene expression data on CGH microarray profiles. *A*, genes in the 7p11-p12 amplicon in the MDA-468 cell line are highly expressed (*red dots*) and include the *EGFR* oncogene. *B*, several genes in the 17q12, 17q21.3, and 17q23 amplicons in the BT-474 breast cancer cell line are highly overexpressed (*red*) and include the *HOXB7* gene. The data labels and color coding are as indicated for Fig. 2*C*. *Insets* show chromosomal CGH profiles for the corresponding chromosomes and validation of the increased copy number by interphase FISH using *EGFR* (*red*) and chromosome 7 centromere probe (*green*) to MDA-468 (*A*) and *HOXB7*-specific probe (*red*) and chromosome 17 centromere (*green*) to BT-474 cells (*B*).

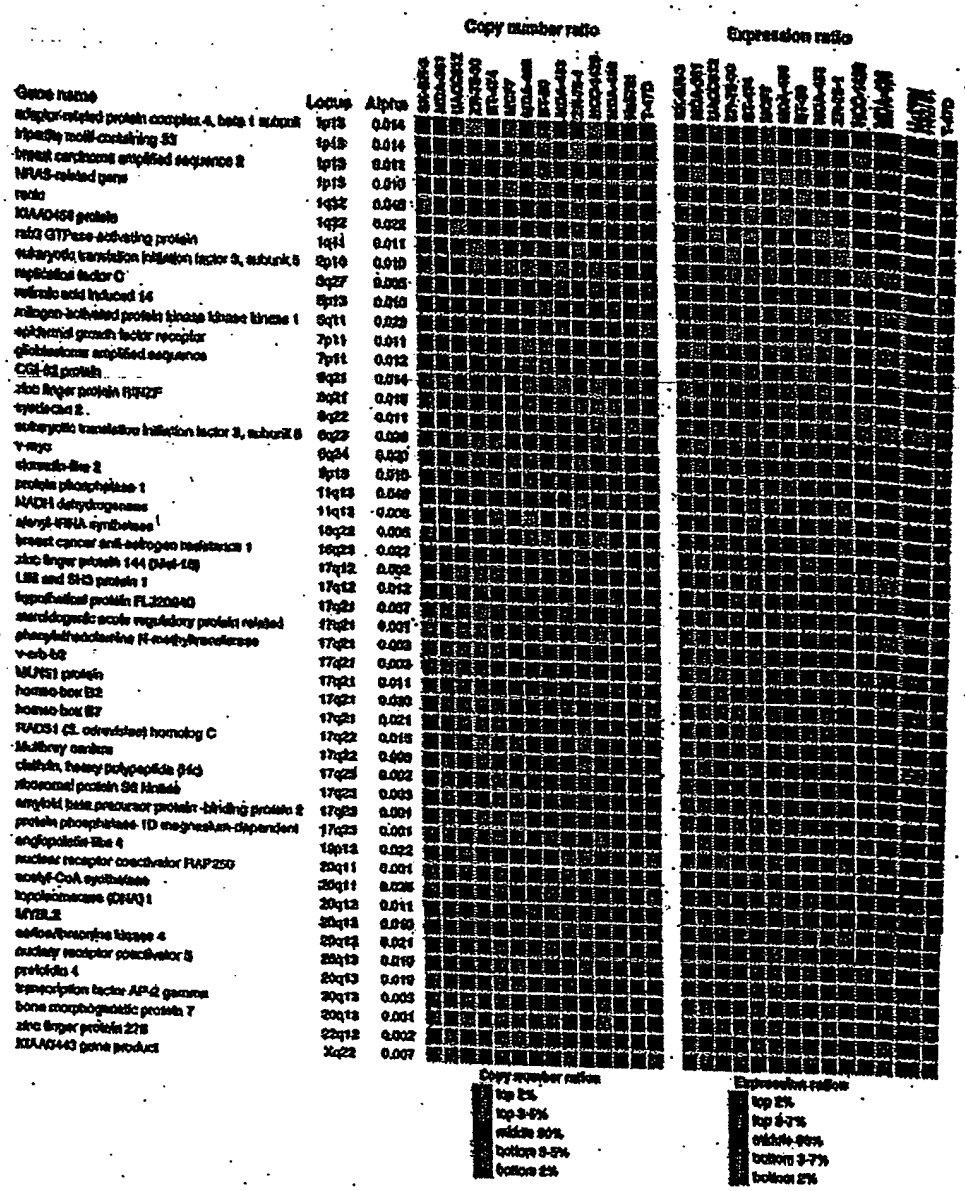


Fig. 4. List of 50 genes with a statistically significant correlation (α value <0.05) between gene copy number and gene expression. Name, chromosomal location, and the α value for each gene are indicated. The genes have been ordered according to their position in the genome. The color maps on the *right* illustrate the copy number and expression ratio patterns in the 14 cell lines. The key to the color code is shown at the *bottom* of the graph. *Gray squares*, missing values. The complete list of 270 genes is shown in supplemental Fig. B.

amplification was validated to be present in 10.2% of 363 primary breast cancers by FISH to a tissue microarray and was associated with poor prognosis of the patients ($P = 0.001$).

Statistical Identification and Characterization of 270 Highly Expressed Genes in Amplicons. Statistical comparison of expression levels of all genes as a function of gene amplification identified 270 genes whose expression was significantly influenced by copy number across all 14 cell lines (Fig. 4, Supplemental Fig. B). According to the gene ontology data,[8] 91 of the 270 genes represented hypothetical proteins or genes with no functional annotation, whereas 179 had associated functional information available. Of these, 151 (84%) are implicated in apoptosis, cell proliferation, signal transduction, and transcription, whereas 28 (16%) had functional annotations that could not be directly linked with cancer.

DISCUSSION

The importance of recurrent gene and chromosome copy number changes in the development and progression of solid tumors has been characterized in >1000 publications applying CGH[9] (9, 10), as well as in a large number of other molecular cytogenetic, cytogenetic, and molecular genetic studies. The effects of these somatic genetic changes on gene expression levels have remained largely unknown, although a few studies have explored gene expression changes occurring in specific amplicons (15, 19–21). Here, we applied genome-wide cDNA microarrays to identify transcripts whose expression changes were attributable to underlying gene copy number alterations in breast cancer.

The overall impact of copy number on gene expression patterns was substantial with the most dramatic effects seen in the case of high-

[8] Internet address: http://www.geneontology.org/.

[9] Internet address: http://www.ncbi.nlm.nih.gov/entrez.

level copy number increase. Low-level copy number gains and losses also had a significant influence on expression levels of genes in the regions affected, but these effects were more subtle on a gene-by-gene basis than those of high-level amplifications. However, the impact of low-level gains on the dysregulation of gene expression patterns in cancer may be equally important if not more important than that of high-level amplifications. Aneuploidy and low-level gains and losses of chromosomal arms represent the most common types of genetic alterations in breast and other cancers and, therefore, have an influence on many genes. Our results in breast cancer extend the recent studies on the impact of aneuploidy on global gene expression patterns in yeast cells, acute myeloid leukemia, and a prostate cancer model system (22–24).

The CGH microarray analysis identified 24 independent breast cancer amplicons. We defined the precise boundaries for many amplicons detected previously by chromosomal CGH (9, 10, 25, 26) and also discovered novel amplicons that had not been detected previously, presumably because of their small size (only 1–2 Mb) or close proximity to other larger amplicons. One of these novel amplicons involved the homeobox gene region at 17q21.3 and led to the overexpression of the *HOXB7* and *HOXB2* genes. The homeodomain transcription factors are known to be key regulators of embryonic development and have been occasionally reported to undergo aberrant expression in cancer (27, 28). *HOXB7* transfection induced cell proliferation in melanoma, breast, and ovarian cancer cells and increased tumorigenicity and angiogenesis in breast cancer (29–32). The present results imply that gene amplification may be a prominent mechanism for overexpressing *HOXB7* in breast cancer and suggest that *HOXB7* contributes to tumor progression and confers an aggressive disease phenotype in breast cancer. This view is supported by our finding of amplification of *HOXB7* in 10% of 363 primary breast cancers, as well as an association of amplification with poor prognosis of the patients.

We carried out a systematic search to identify genes whose expression levels across all 14 cell lines were attributable to amplification status. Statistical analysis revealed 270 such genes (representing ~2% of all genes on the array), including not only previously described amplified genes, such as *HER-2*, *MYC*, *EGFR*, ribosomal protein s6 kinase, and *AIB3*, but also numerous novel genes such as *NRAS-related gene* (1p13), *syndecan-2* (8q22), and *bone morphogenic protein* (20q13.1), whose activation by amplification may similarly promote breast cancer progression. Most of the 270 genes have not been implicated previously in breast cancer development and suggest novel pathogenetic mechanisms. Although we would not expect all of them to be causally involved, it is intriguing that 84% of the genes with associated functional information were implicated in apoptosis, cell proliferation, signal transduction, transcription, or other cellular processes that could directly imply a possible role in cancer progression. Therefore, a detailed characterization of these genes may provide biological insights to breast cancer progression and might lead to the development of novel therapeutic strategies.

In summary, we demonstrate application of cDNA microarrays to the analysis of both copy number and expression levels of over 12,000 transcripts throughout the breast cancer genome, roughly once every 267 kb. This analysis provided: (*a*) evidence of a prominent global influence of copy number changes on gene expression levels; (*b*) a high-resolution map of 24 independent amplicons in breast cancer; and (*c*) identification of a set of 270 genes, the overexpression of which was statistically attributable to gene amplification. Characterization of a novel amplicon at 17q21.3 implicated amplification and overexpression of the *HOXB7* gene in breast cancer, including a clinical association between *HOXB7* amplification and poor patient prognosis. Overall, our results illustrate how the identification of genes activated by gene amplification provides a powerful approach to highlight genes with an important role in cancer as well as to prioritize and validate putative targets for therapy development.

REFERENCES

1. Golub, T. R., Slonim, D. K., Tamayo, P., Huard, C., Gaasenbeek, M., Mesirov, J. P., Coller, H., Loh, M. L., Downing, J. R., Caligiuri, M. A., Bloomfield, C. D., and Lander, E. S. Molecular classification of cancer: class discovery and class prediction by gene expression monitoring. Science (Wash. DC), *286:* 531–537, 1999.
2. Alizadeh, A. A., Eisen, M. B., Davis, R. E., Ma, C., Lossos, I. S., Rosenwald, A., Boldrick, J. C., Sabet, H., Tran, T., Yu, X., *et al.* Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. Nature (Lond.), *403:* 503–511, 2000.
3. Bittner, M., Meltzer, P., Chen, Y., Jiang, Y., Seftor, E., Hendrix, M., Radmacher, M., Simon, R., Yakhini, Z., Ben-Dor, A., *et al.* Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature (Lond.), *406:* 536–540, 2000.
4. Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., *et al.* Molecular portraits of human breast tumours. Nature (Lond.), *406:* 747–752, 2000.
5. Dhanasekaran, S. M., Barrette, T. R., Ghosh, D., Shah, R., Varambally, S., Kurachi, K., Pienta, K. J., Rubin, M. A., and Chinnaiyan, A. M. Delineation of prognostic biomarkers in prostate cancer. Nature (Lond.), *412:* 822–826, 2001.
6. Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., *et al.* Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc. Natl. Acad. Sci. USA, *98:* 10869–10874, 2001.
7. Ross, J. S., and Fletcher, J. A. The *HER-2/neu* oncogene: prognostic factor, predictive factor and target for therapy. Semin. Cancer Biol., *9:* 125–138, 1999.
8. Arteaga, C. L. The epidermal growth factor receptor: from mutant oncogene in nonhuman cancers to therapeutic target in human neoplasia. J. Clin. Oncol., *19:* 32–40, 2001.
9. Knuutila, S., Bjorkqvist, A. M., Autio, K., Tarkkanen, M., Wolf, M., Monni, O., Szymanska, J., Larramendy, M. L., Tapper, J., Pere, H., El-Rifai, W., *et al.* DNA copy number amplifications in human neoplasms: review of comparative genomic hybridization studies. Am. J. Pathol., *152:* 1107–1123, 1998.
10. Knuutila S., Autio K., and Aalto Y. Online access to CGH data of DNA sequence copy number changes. Am. J. Pathol., *157:* 689, 2000.
11. DeRisi, J., Penland, L., Brown, P. O., Bittner, M. L., Meltzer, P. S., Ray, M., Chen, Y., Su, Y. A., and Trent, J. M. Use of a cDNA microarray to analyse gene expression patterns in human cancer. Nat. Genet., *14:* 457–460, 1996.
12. Shalon, D., Smith, S. J., and Brown, P. O. A DNA microarray system for analyzing complex DNA samples using two-color fluorescent probe hybridization. Genome Res., *6:* 639–645, 1996.
13. Mousses, S., Bittner, M. L., Chen, Y., Dougherty, E. R., Baxevanis, A., Meltzer, P. S., and Trent, J. M. Gene expression analysis by cDNA microarrays. *In:* R. J. Livesey and S. P. Hunt (eds.), Functional Genomics, pp. 113–137. Oxford: Oxford University Press, 2000.
14. Pollack, J. R., Perou, C. M., Alizadeh, A. A., Eisen, M. B., Pergamenschikov, A., Williams, C. F., Jeffrey, S. S., Botstein, D., and Brown, P. O. Genome-wide analysis of DNA copy-number changes using cDNA microarrays. Nat. Genet., *23:* 41–46, 1999.
15. Monni, O., Bärlund, M., Mousses, S., Kononen, J., Sauter, G., Heiskanen, M., Paavola, P., Avela, K., Chen, Y., Bittner, M. L., and Kallioniemi, A. Comprehensive copy number and gene expression profiling of the 17q23 amplicon in human breast cancer. Proc. Natl. Acad. Sci. USA, *98:* 5711–5716, 2001.
16. Chen, Y., Dougherty, E. R., and Bittner, M. L. Ratio-based decisions and the quantitative analysis of cDNA microarray images. J. Biomed. Optics, *2:* 364–374, 1997.
17. Bärlund, M., Forozan, F., Kononen, J., Bubendorf, L., Chen, Y., Bittner, M. L., Torhorst, J., Haas, P., Bucher, C., Sauter, G., *et al.* Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J. Natl. Cancer Inst., *92:* 1252–1259, 2000.
18. Andersen, C. L., Hostetter, G., Grigoryan, A., Sauter, G., and Kallioniemi, A. Improved procedure for fluorescence *in situ* hybridization on tissue microarrays. Cytometry, *45:* 83–86, 2001.
19. Kauraniemi, P., Bärlund, M., Monni, O., and Kallioniemi, A. New amplified and highly expressed genes discovered in the ERBB2 amplicon in breast cancer by cDNA microarrays. Cancer Res., *61:* 8235–8240, 2001.
20. Clark, J., Edwards, S., John, M., Flohr, P., Gordon, T., Maillard, K., Giddings, I., Brown, C., Bagherzadeh, A., Campbell, C., Shipley, J., Wooster, R., and Cooper, C. S. Identification of amplified and expressed genes in breast cancer by comparative hybridization onto microarrays of randomly selected cDNA clones. Genes Chromosomes Cancer, *34:* 104–114, 2002.
21. Varis, A., Wolf, M., Monni, O., Vakkari, M. L., Kokkola, A., Moskaluk, C., Frierson, H., Powell, S. M., Knuutila, S., Kallioniemi, A., and El-Rifai, W. Targets of gene amplification and overexpression at 17q in gastric cancer. Cancer Res., *62:* 2625–2629, 2002.
22. Hughes, T. R., Roberts, C. J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D., Burchard, J., Dow, S., Ward, T. R., Kidd, M. J., Friend, S. H., and Marton M. J.

Widespread aneuploidy revealed by DNA microarray expression profiling. Nat. Genet., *25:* 333–337, 2000.

23. Virtaneva, K., Wright, F. A., Tanner, S. M., Yuan, B., Lemon, W. J., Caligiuri, M. A., Bloomfield, C. D., de La Chapelle, A., and Krahe, R. Expression profiling reveals fundamental biological differences in acute myeloid leukemia with isolated trisomy 8 and normal cytogenetics. Proc. Natl. Acad. Sci. USA, *98:* 1124–1129, 2001.
24. Phillips, J. L., Hayward, S. W., Wang, Y., Vasselli, J., Pavlovich, C., Padilla-Nash, H., Pezullo, J. R., Ghadimi, B. M., Grossfeld, G. D., Rivera, A., Linehan, W. M., Cunha, G. R., and Ried, T. The consequences of chromosomal aneuploidy on gene expression profiles in a cell line model for prostate carcinogenesis. Cancer Res., *61:* 8143–8149, 2001.
25. Bärlund, M., Tirkkonen, M., Forozan, F., Tanner, M. M., Kallioniemi, O. P., and Kallioniemi, A. Increased copy number at 17q22–q24 by CGH in breast cancer is due to high-level amplification of two separate regions. Genes Chromosomes Cancer, *20:* 372–376, 1997.
26. Tamer, M. M., Tirkkonen, M., Kallioniemi, A., Isola, J., Kuukasjärvi, T., Collins, C., Kowbel, D., Guan, X. Y., Trent, J., Gray, J. W., Meltzer, P., and Kallioniemi O. P. Independent amplification and frequent co-amplification of three nonsyntenic regions on the long arm of chromosome 20 in human breast cancer. Cancer Res., *56:* 3441–3445, 1996.
27. Cillo, C., Faiella, A., Cantile, M., and Boncinelli, E. Homeobox genes and cancer. Exp. Cell Res., *248:* 1–9, 1999.
28. Cillo, C., Cantile, M., Faiella, A., and Boncinelli, E. Homeobox genes in normal and malignant cells. J. Cell. Physiol., *188:* 161–169, 2001.
29. Care, A., Silvani, A., Meccia, E., Mattia, G., Stoppacciaro, A., Parmiani, G., Peschle, C., and Colombo, M. P. HOXB7 constitutively activates basic fibroblast growth factor in melanomas. Mol. Cell. Biol., *16:* 4842–4851, 1996.
30. Care, A., Silvani, A., Meccia, E., Mattia, G., Peschle, C., and Colombo, M. P. Transduction of the SkBr3 breast carcinoma cell line with the *HOXB7* gene induces bFGF expression, increases cell proliferation and reduces growth factor dependence. Oncogene, *16:* 3285–3289, 1998.
31. Care, A., Felicetti, F., Meccia, E., Bottero, L., Parenza, M., Stoppacciaro, A., Peschle, C., and Colombo, M. P. HOXB7: a key factor for tumor-associated angiogenic switch. Cancer Res., *61:* 6532–6539, 2001.
32. Naora, H., Yang, Y. Q., Montz, F. J., Seidman, J. D., Kurman, R. J., and Roden, R. B. A serologically identified tumor antigen encoded by a homeobox gene promotes growth of ovarian epithelial cells. Proc. Natl. Acad. Sci. USA, *98:* 4060–4065, 2001.

Microarray analysis reveals a major direct role of DNA copy number alteration in the transcriptional program of human breast tumors

Jonathan R. Pollack*†‡, Therese Sørlie§, Charles M. Perou¶, Christian A. Rees‖**, Stefanie S. Jeffrey††, Per E. Lonning‡‡, Robert Tibshirani§§, David Botstein‖, Anne-Lise Børresen-Dale§, and Patrick O. Brown†¶¶

Departments of *Pathology, ‖Genetics, ††Surgery, §§Health Research and Policy, and ¶¶Biochemistry, and †Howard Hughes Medical Institute, Stanford University School of Medicine, Stanford, CA 94305; §Department of Genetics, Norwegian Radium Hospital, Montebello, N-0310 Oslo, Norway; ‡‡Department of Medicine (Oncology), Haukeland University Hospital, N-5021 Bergen, Norway; and ¶Department of Genetics and Lineberger Comprehensive Cancer Center, University of North Carolina, Chapel Hill, NC 27599

Contributed by Patrick O. Brown, August 6, 2002

Genomic DNA copy number alterations are key genetic events in the development and progression of human cancers. Here we report a genome-wide microarray comparative genomic hybridization (array CGH) analysis of DNA copy number variation in a series of primary human breast tumors. We have profiled DNA copy number alteration across 6,691 mapped human genes, in 44 predominantly advanced, primary breast tumors and 10 breast cancer cell lines. While the overall patterns of DNA amplification and deletion corroborate previous cytogenetic studies, the high-resolution (gene-by-gene) mapping of amplicon boundaries and the quantitative analysis of amplicon shape provide significant improvement in the localization of candidate oncogenes. Parallel microarray measurements of mRNA levels reveal the remarkable degree to which variation in gene copy number contributes to variation in gene expression in tumor cells. Specifically, we find that 62% of highly amplified genes show moderately or highly elevated expression, that DNA copy number influences gene expression across a wide range of DNA copy number alterations (deletion, low-, mid- and high-level amplification), that on average, a 2-fold change in DNA copy number is associated with a corresponding 1.5-fold change in mRNA levels, and that overall, at least 12% of all the variation in gene expression among the breast tumors is directly attributable to underlying variation in gene copy number. These findings provide evidence that widespread DNA copy number alteration can lead directly to global deregulation of gene expression, which may contribute to the development or progression of cancer.

Conventional cytogenetic techniques, including comparative genomic hybridization (CGH) (1), have led to the identification of a number of recurrent regions of DNA copy number alteration in breast cancer cell lines and tumors (2–4). While some of these regions contain known or candidate oncogenes [e.g., FGFR1 (8p11), MYC (8q24), CCND1 (11q13), ERBB2 (17q12), and ZNF217 (20q13)] and tumor suppressor genes [RB1 (13q14) and TP53 (17p13)], the relevant gene(s) within other regions (e.g., gain of 1q, 8q22, and 17q22–24, and loss of 8p) remain to be identified. A high-resolution genome-wide map, delineating the boundaries of DNA copy number alterations in tumors, should facilitate the localization and identification of oncogenes and tumor suppressor genes in breast cancer. In this study, we have created such a map, using array-based CGH (5–7) to profile DNA copy number alteration in a series of breast cancer cell lines and primary tumors.

An unresolved question is the extent to which the widespread DNA copy number changes that we and others have identified in breast tumors alter expression of genes within involved regions. Because we had measured mRNA levels in parallel in the same samples (8), using the same DNA microarrays, we had an opportunity to explore on a genomic scale the relationship between DNA copy number changes and gene expression. From this analysis, we have identified a significant impact of widespread DNA copy number alteration on the transcriptional programs of breast tumors.

Materials and Methods

Tumors and Cell Lines. Primary breast tumors were predominantly large (>3 cm), intermediate-grade, infiltrating ductal carcinomas, with more than 50% being lymph node positive. The fraction of tumor cells within specimens averaged at least 50%. Details of individual tumors have been published (8, 9), and are summarized in Table 1, which is published as supporting information on the PNAS web site, www.pnas.org. Breast cancer cell lines were obtained from the American Type Culture Collection. Genomic DNA was isolated either using Qiagen genomic DNA columns, or by phenol/chloroform extraction followed by ethanol precipitation.

DNA Labeling and Microarray Hybridizations. Genomic DNA labeling and hybridizations were performed essentially as described in Pollack *et al.* (7), with slight modifications. Two micrograms of DNA was labeled in a total volume of 50 microliters and the volumes of all reagents were adjusted accordingly. "Test" DNA (from tumors and cell lines) was fluorescently labeled (Cy5) and hybridized to a human cDNA microarray containing 6,691 different mapped human genes (i.e., UniGene clusters). The "reference" (labeled with Cy3) for each hybridization was normal female leukocyte DNA from a single donor. The fabrication of cDNA microarrays and the labeling and hybridization of mRNA samples have been described (8).

Data Analysis and Map Positions. Hybridized arrays were scanned on a GenePix scanner (Axon Instruments, Foster City, CA), and fluorescence ratios (test/reference) calculated using SCANALYZE software (available at http://rana.lbl.gov). Fluorescence ratios were normalized for each array by setting the average log fluorescence ratio for all array elements equal to 0. Measurements with fluorescence intensities more than 20% above background were considered reliable. DNA copy number profiles that deviated significantly from background ratios measured in normal genomic DNA control hybridizations were interpreted as evidence of real DNA copy number alteration (see *Estimating Significance of Altered Fluorescence Ratios* in the supporting information). When indicated, DNA copy number profiles are displayed as a moving average (symmetric 5-nearest neighbors). Map positions for arrayed human cDNAs were assigned by

Abbreviation: CGH, comparative genomic hybridization.

†To whom reprint requests should be addressed at: Department of Pathology, Stanford University School of Medicine, CCSR Building, Room 3245A, 269 Campus Drive, Stanford, CA 94305-5176. E-mail: pollack1@stanford.edu.

**Present address: Zyomyx Inc., Hayward, CA 94545.

www.pnas.org/cgi/doi/10.1073/pnas.162471999

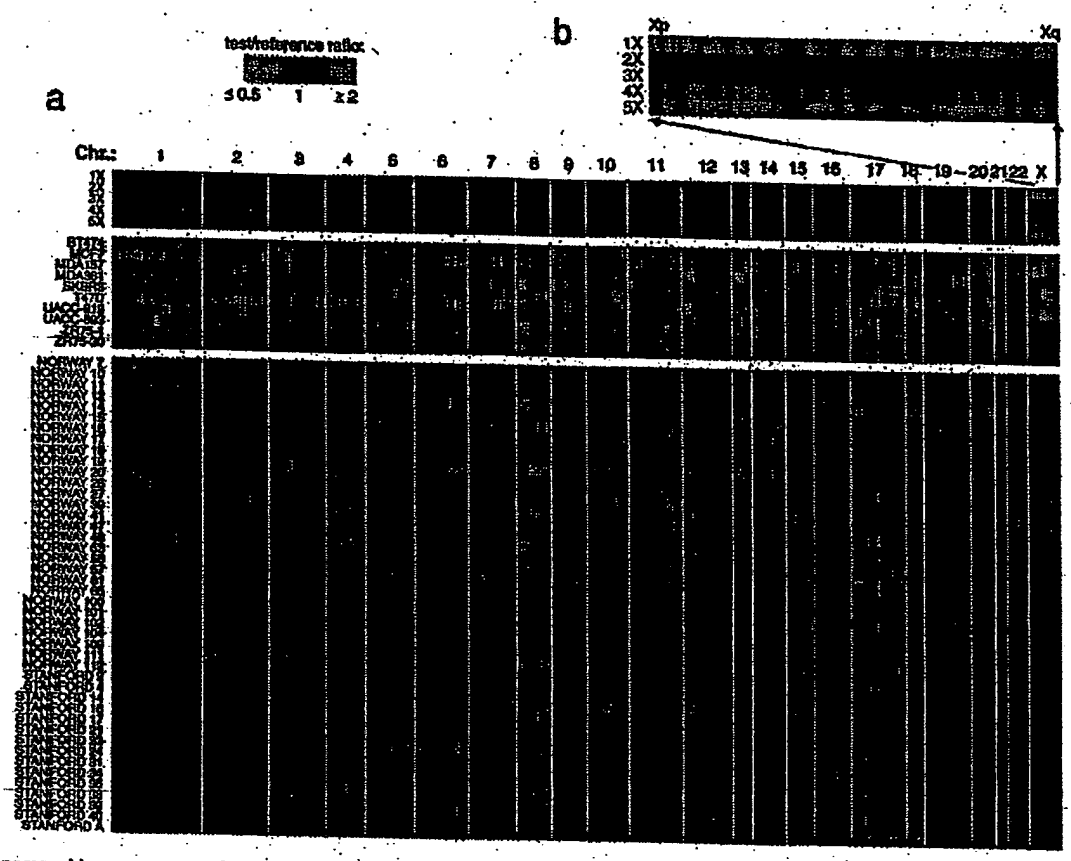


Fig. 1. Genome-wide measurement of DNA copy number alteration by array CGH. (*a*) DNA copy number profiles are illustrated for cell lines containing different numbers of X chromosomes, for breast cancer cell lines, and for breast tumors. Each row represents a different cell line or tumor, and each column represents one of 6,691 different mapped human genes present on the microarray, ordered by genome map position from 1pter through Xqter. Moving average (symmetric 5-nearest neighbors) fluorescence ratios (test/reference) are depicted using a $\log_2$-based pseudocolor scale (indicated), such that red luminescence reflects fold-amplification, green luminescence reflects fold-deletion, and black indicates no change (gray indicates poorly measured data). (*b*) Enlarged view of DNA copy number profiles across the X chromosome, shown for cell lines containing different numbers of X chromosomes.

identifying the starting position of the best and longest match of any DNA sequence represented in the corresponding UniGene cluster (10) against the "Golden Path" genome assembly (http://genome.ucsc.edu/; Oct 7, 2000 Freeze). For UniGene clusters represented by multiple arrayed elements, mean fluorescence ratios (for all elements representing the same UniGene cluster) are reported. For mRNA measurements, fluorescence ratios are "mean-centered" (i.e., reported relative to the mean ratio across the 44 tumor samples). The data set described here can be accessed in its entirety in the supporting information.

Results

We performed CGH on 44 predominantly locally advanced, primary breast tumors and 10 breast cancer cell lines, using cDNA microarrays containing 6,691 different mapped human genes (Fig. 1*a*; also see *Materials and Methods* for details of microarray hybridizations). To take full advantage of the improved spatial resolution of array CGH, we ordered (fluorescence ratios for) the 6,691 cDNAs according to the "Golden Path" (http://genome.ucsc.edu/) genome assembly of the draft human genome sequences (11). In so doing, arrayed cDNAs not only themselves represent genes of potential interest (e.g., candidate oncogenes within amplicons), but also provide precise genetic landmarks for chromosomal regions of amplification and deletion. Parallel analysis of DNA from cell lines containing different numbers of X chromosomes (Fig. 1*b*), as we did before (7), demonstrated the sensitivity of our method to detect single-copy loss (45, XO), and 1.5- (47,XXX), 2- (48,XXXX), or 2.5-fold (49,XXXXX) gains (also see Fig. 5, which is published as supporting information on the PNAS web site). Fluorescence ratios were linearly proportional to copy number ratios, which were slightly underestimated, in agreement with previous observations (7). Numerous DNA copy number alterations were evident in both the breast cancer cell lines and primary tumors (Fig. 1*a*), detected in the tumors despite the presence of euploid non-tumor cell types; the magnitudes of the observed changes were generally lower in the tumor samples. DNA copy-number alterations were found in every cancer cell line and tumor, and on every human chromosome in at least one sample. Recurrent regions of DNA copy number gain and loss were readily identifiable. For example, gains within 1q, 8q, 17q, and 20q were observed in a high proportion of breast cancer cell lines/tumors (90%/69%, 100%/47%, 100%/60%, and 90%/44%, respectively), as were losses within 1p, 3p, 8p, and 13q (80%/24%, 80%/22%, 80%/22%, and 70%/18%, respectively), consistent with published cytogenetic studies (refs. 2–4; a complete listing of gains/losses is provided in Tables 2 and 3, which are published as supporting information on the PNAS web site). The total

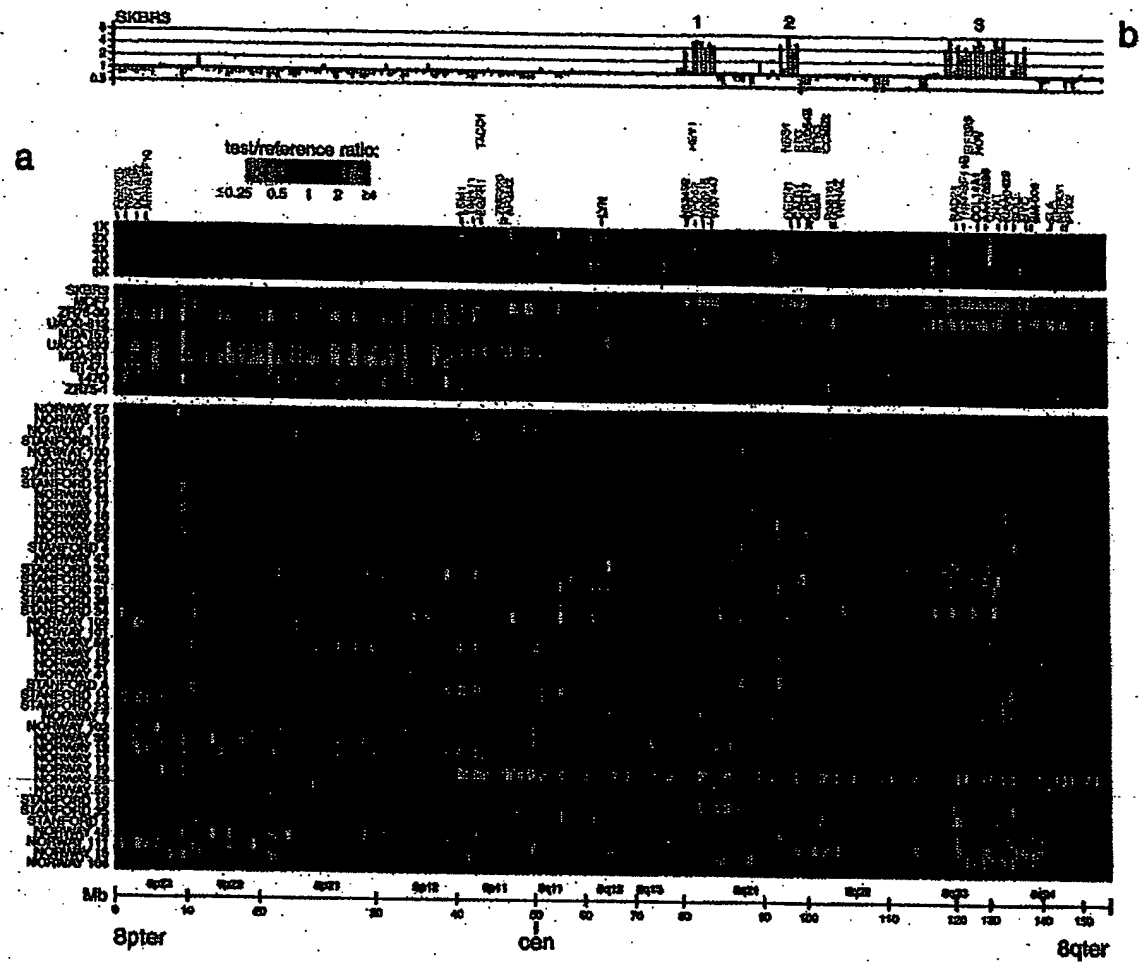


Fig. 2. DNA copy number alteration across chromosome 8 by array CGH. (*a*) DNA copy number profiles are illustrated for cell lines containing different numbers of X chromosomes, for breast cancer cell lines, and for breast tumors. Breast cancer cell lines and tumors are separately ordered by hierarchical clustering to highlight recurrent copy number changes. The 241 genes present on the microarrays and mapping to chromosome 8 are ordered by position along the chromosome. Fluorescence ratios (test/reference) are depicted by a $\log_2$ pseudocolor scale (indicated). Selected genes are indicated with color-coded text (red, increased; green, decreased; black, no change; gray, not well measured) to reflect correspondingly altered mRNA levels (observed in the majority of the subset of samples displaying the DNA copy number change). The map positions for genes of interest that are not represented on the microarray are indicated in the row above those genes represented on the array. (*b*) Graphical display of DNA copy number profile for breast cancer cell line SKBR3. Fluorescence ratios (tumor/normal) are plotted on a $\log_2$ scale for chromosome 8 genes, ordered along the chromosome.

number of genomic alterations (gains and losses) was found to be significantly higher in breast tumors that were high grade ($P = 0.008$), consistent with published CGH data (3), estrogen receptor negative ($P = 0.04$), and harboring TP53 mutations ($P = 0.0006$) (see Table 4, which is published as supporting information on the PNAS web site).

The improved spatial resolution of our array CGH analysis is illustrated for chromosome 8, which displayed extensive DNA copy number alteration in our series. A detailed view of the variation in the copy number of 241 genes mapping to chromosome 8 revealed multiple regions of recurrent amplification; each of these potentially harbors a different known or previously uncharacterized oncogene (Fig. 2*a*). The complexity of amplicon structure is most easily appreciated in the breast cancer cell line SKBR3. Although a conventional CGH analysis of 8q in SKBR3 identified only two distinct regions of amplification (12), we observed three distinct regions of high-level amplification (labeled 1–3 in Fig. 2*b*). For each of these regions we can define the boundaries of the interval recurrently amplified in the tumors we examined; in each case, known or plausible candidate oncogenes can be identified (a description of these regions, as well as the recurrently amplified regions on chromosomes 17 and 20, can be found in Figs. 6 and 7, which are published as supporting information on the PNAS web site).

For a subset of breast cancer cell lines and tumors (4 and 37, respectively), and a subset of arrayed genes (6,095), mRNA levels were quantitatively measured in parallel by using cDNA microarrays (8). The parallel assessment of mRNA levels is useful in the interpretation of DNA copy number changes. For example, the highly amplified genes that are also highly expressed are the strongest candidate oncogenes within an amplicon. Perhaps more significantly, our parallel analysis of DNA copy number changes and mRNA levels provides us the opportunity to assess the global impact of widespread DNA copy number alteration on gene expression in tumor cells.

A strong influence of DNA copy number on gene expression is evident in an examination of the pseudocolor representations

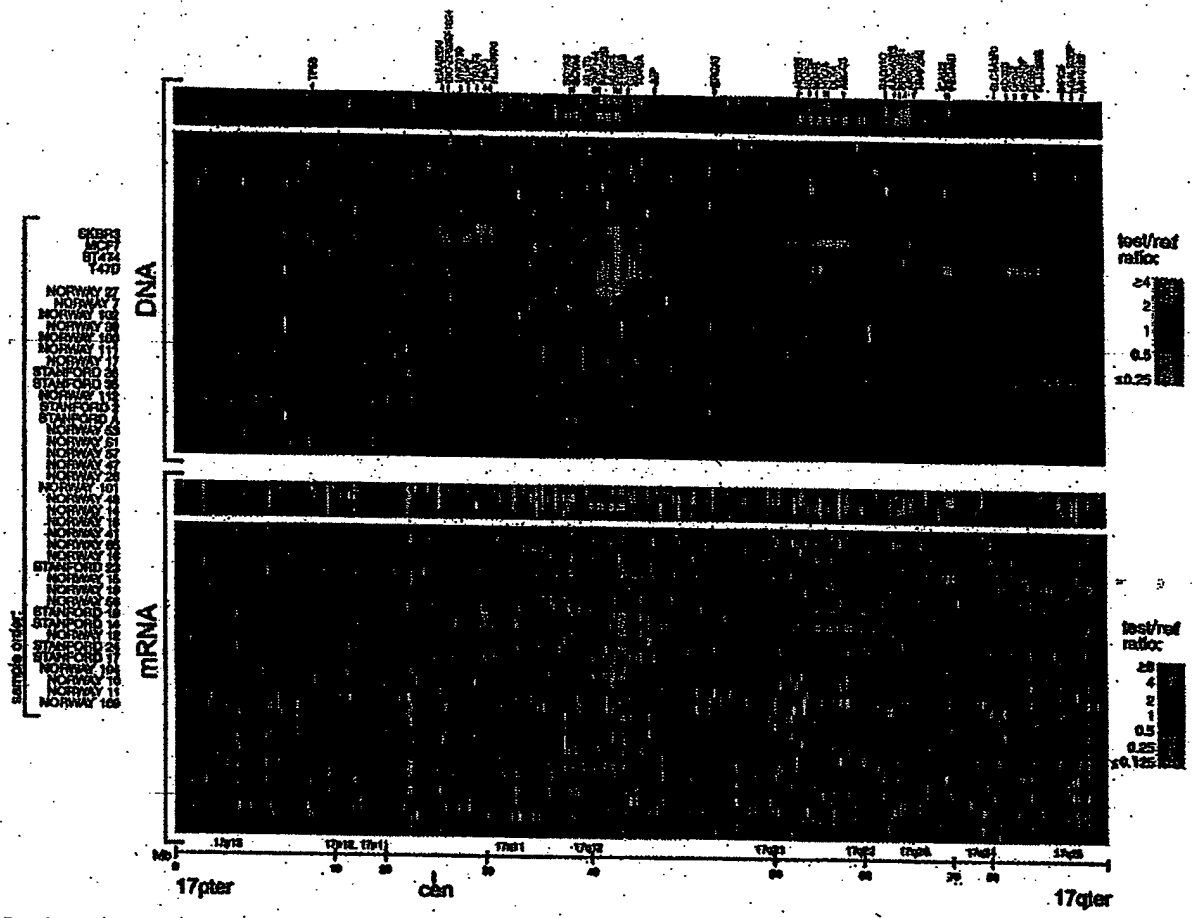


Fig. 3. Concordance between DNA copy number and gene expression across chromosome 17. DNA copy number alteration (*Upper*) and mRNA levels (*Lower*) are illustrated for breast cancer cell lines and tumors. Breast cancer cell lines and tumors are separately ordered by hierarchical clustering (*Upper*), and the identical sample order is maintained (*Lower*). The 354 genes present on the microarrays and mapping to chromosome 17, and for which both DNA copy number and mRNA levels were determined, are ordered by position along the chromosome; selected genes are indicated in color-coded text (see Fig. 2 legend). Fluorescence ratios (test/reference) are depicted by separate $\log_2$ pseudocolor scales (indicated).

of DNA copy number and mRNA levels for genes on chromosome 17 (Fig. 3). The overall patterns of gene amplification and elevated gene expression are quite concordant; i.e., a significant fraction of highly amplified genes appear to be correspondingly highly expressed. The concordance between high-level amplification and increased gene expression is not restricted to chromosome 17. Genome-wide, of 117 high-level DNA amplifications (fluorescence ratios >4, and representing 91 different genes), 62% (representing 54 different genes; see Table 5, which is published as supporting information on the PNAS web site) are found associated with at least moderately elevated mRNA levels (mean-centered fluorescence ratios >2), and 42% (representing 36 different genes) are found associated with comparably highly elevated mRNA levels (mean-centered fluorescence ratios >4).

To determine the extent to which DNA deletion and lower-level amplification (in addition to high-level amplification) are also associated with corresponding alterations in mRNA levels, we performed three separate analyses on the complete data set (4 cell lines and 37 tumors, across 6,095 genes). First, we determined the average mRNA levels for each of five classes of genes, representing DNA deletion, no change, and low-, medium-, and high-level amplification (Fig. 4*a*). For both the breast cancer cell lines and tumors, average mRNA levels tracked with DNA copy number across all five classes, in a statistically significant fashion (P values for pair-wise Student's t tests comparing adjacent classes: cell lines, 4×10^{-49}, 1×10^{-49}, 5×10^{-5}, 1×10^{-2}; tumors, 1×10^{-43}, 1×10^{-214}, 5×10^{-41}, 1×10^{-4}). A linear regression of the average log(DNA copy number), for each class, against average log(mRNA level) demonstrated that on average, a 2-fold change in DNA copy number was accompanied by 1.4- and 1.5-fold changes in mRNA level for the breast cancer cell lines and tumors, respectively (Fig. 4*a*, regression line not shown). Second, we characterized the distribution of the 6,095 correlations between DNA copy number and mRNA level, each across the 37 tumor samples (Fig. 4*b*). The distribution of correlations forms a normal-shaped curve, but with the peak markedly shifted in the positive direction from zero. This shift is statistically significant, as evidenced in a plot of observed vs. expected correlations (Fig. 4*c*), and reflects a pervasive global influence of DNA copy number alterations on gene expression. Notably, the highest correlations between DNA copy number and mRNA level (the right tail of the distribution in Fig. 4*b*) comprise both amplified and deleted genes (data not shown). Third, we used a linear regression model to estimate the fraction of all variation measured in mRNA levels among the 37

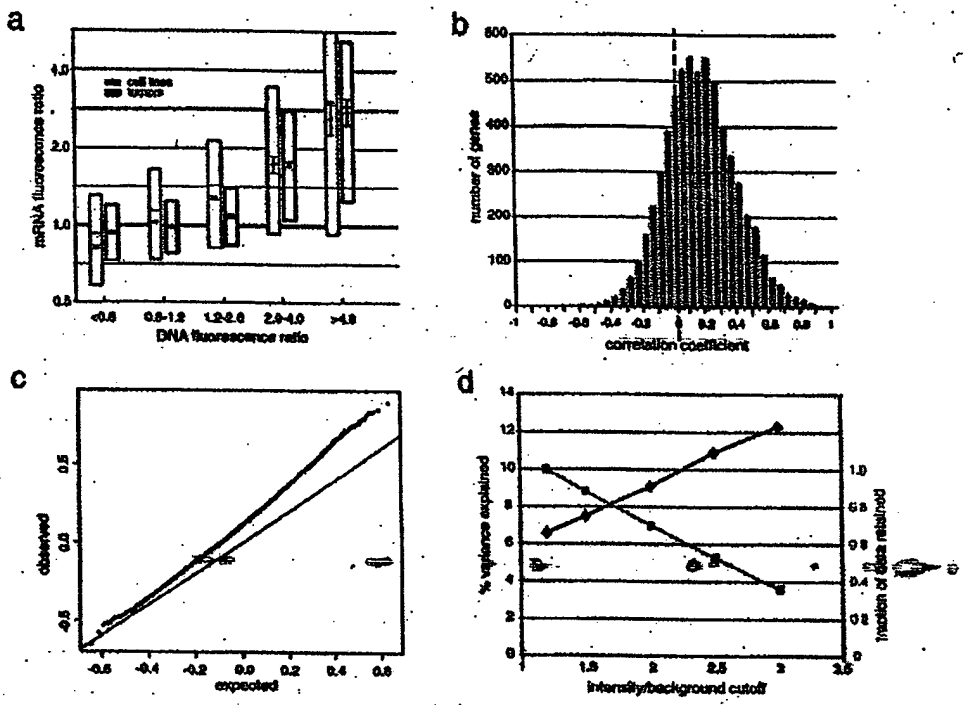


Fig. 4. Genome-wide influence of DNA copy number alterations on mRNA levels. (*a*) For breast cancer cell lines (gray) and tumor samples (black), both mean-centered mRNA fluorescence ratio (log_2 scale) quartiles (box plots indicate 25th, 50th, and 75th percentile) and averages (diamonds; *Y*-value error bars indicate standard errors of the mean) are plotted for each of five classes of genes, representing DNA deletion (tumor/normal ratio < 0.8), no change (0.8–1.2), low- (1.2–2), medium- (2–4), and high-level (>4) amplification. *P* values for pair-wise Student's *t* tests, comparing averages between adjacent classes (moving left to right), are 4×10^{-43}, 1×10^{-49}, 5×10^{-5}, 1×10^{-3} (cell lines), and 1×10^{-43}, 1×10^{-214}, 5×10^{-41}, 1×10^{-4} (tumors). (*b*) Distribution of correlations between DNA copy number and mRNA levels, for 6,095 different human genes across 37 breast tumor samples. (*c*) Plot of observed versus expected correlation coefficients. The expected values were obtained by randomization of the sample labels in the DNA copy number data set. The line of unity is indicated. (*d*) Percent variance in gene expression (among tumors) directly explained by variation in gene copy number. Percent variance explained (black line) and fraction of data retained (gray line) are plotted for different fluorescence intensity/background (a rough surrogate for signal/noise) cutoff values. Fraction of data retained is relative to the 1.2 intensity/background cutoff. Details of the linear regression model used to estimate the fraction of variation in gene expression attributable to underlying DNA copy number alteration can be found in the supporting information (see *Estimating the Fraction of Variation in Gene Expression Attributable to Underlying DNA Copy Number Alteration*).

tumors that could be attributed to underlying variation in DNA copy number. From this analysis, we estimate that, overall, about 7% of all of the observed variation in mRNA levels can be explained directly by variation in copy number of the altered genes (Fig. 4*d*). We can reduce the effects of experimental measurement error on this estimate by using only that fraction of the data most reliably measured (fluorescence intensity/background >3); using that data, our estimate of the percent variation in mRNA levels directly attributed to variation in gene copy number increases to 12% (Fig. 4*d*). This still undoubtedly represents a significant underestimate, as the observed variation in global gene expression is affected not only by true variation in the expression programs of the tumor cells themselves, but also by the variable presence of non-tumor cell types within clinical samples.

Discussion

This genome-wide, array CGH analysis of DNA copy number alteration in a series of human breast tumors demonstrates the usefulness of defining amplicon boundaries at high resolution (gene-by-gene), and quantitatively measuring amplicon shape, to assist in locating and identifying candidate oncogenes. By analyzing mRNA levels in parallel, we have also discovered that changes in DNA copy number have a large, pervasive, direct effect on global gene expression patterns in both breast cancer cell lines and tumors. Although the DNA microarrays used in our analysis may display a bias toward characterized and/or highly expressed genes, because we are examining such a large fraction of the genome (approximately 20% of all human genes), and because, as detailed above, we are likely underestimating the contribution of DNA copy number changes to altered gene expression, we believe our findings are likely to be generalizable (but would nevertheless still be remarkable if only applicable to this set of ~6,100 genes).

In budding yeast, aneuploidy has been shown to result in chromosome-wide gene expression biases (13). Two recent studies have begun to examine the global relationship between DNA copy number and gene expression in cancer cells. In agreement with our findings, Phillips *et al.* (14) have shown that with the acquisition of tumorigenicity in an immortalized prostate epithelial cell line, new chromosomal gains and losses resulted in a statistically significant respective increase and decrease in the average expression level of involved genes. In contrast, Platzer *et al.* (15) recently reported that in metastatic colon tumors only ~4% of genes within amplified regions were found more highly (>2-fold) expressed, when compared with normal colonic epithelium. This report differs substantially from our finding that 62% of highly amplified genes in breast cancer exhibit at least 2-fold increased expression. These contrasting findings may reflect methodological differences between the

studies. For example, the study of Platzer *et al.* (15) may have systematically under-measured gene expression changes. In this regard it is remarkable that only 14 transcripts of many thousand residing within unamplified chromosomal regions were found to exhibit at least 4-fold altered expression in metastatic colon cancer. Additionally, their reliance on lower-resolution chromosomal CGH may have resulted in poorly delimiting the boundaries of high-complexity amplicons, effectively overcalling regions with amplification. Alternatively, the contrasting findings for amplified genes may represent real biological differences between breast and metastatic colon tumors; resolution of this issue will require further studies.

Our finding that widespread DNA copy number alteration has a large, pervasive and direct effect on global gene expression patterns in breast cancer has several important implications. First, this finding supports a high degree of copy number-dependent gene expression in tumors. Second, it suggests that most genes are not subject to specific autoregulation or dosage compensation. Third, this finding cautions that elevated expression of an amplified gene cannot alone be considered strong independent evidence of a candidate oncogene's role in tumorigenesis. In our study, fully 62% of highly amplified genes demonstrated moderately or highly elevated expression. This highlights the importance of high-resolution mapping of amplicon boundaries and shape [to identify the "driving" gene(s) within amplicons (16)], on a large number of samples, in addition to functional studies. Fourth, this finding suggests that analyzing the genomic distribution of expressed genes, even within existing microarray gene expression data sets, may permit the inference of DNA copy number aberration, particularly aneuploidy (where gene expression can be averaged across large chromosomal regions; see Fig. 3 and supporting information). Fifth, this finding implies that a substantial portion of the phenotypic uniqueness (and by extension, the heterogeneity in clinical behavior) among patients' tumors may be traceable to underlying variation in DNA copy number. Sixth, this finding supports a possible role for widespread DNA copy number alteration in tumorigenesis (17, 18), beyond the amplification of specific oncogenes and deletion of specific tumor suppressor genes. Widespread DNA copy number alteration, and the concomitant widespread imbalance in gene expression, might disrupt critical stochiometric relationships in cell metabolism and physiology (e.g., proteosome, mitotic spindle), possibly promoting further chromosomal instability and directly contributing to tumor development or progression. Finally, our findings suggest the possibility of cancer therapies that exploit specific or global imbalances in gene expression in cancer.

We thank the many members of the P.O.B. and D.B. labs for helpful discussions. J.R.P. was a Howard Hughes Medical Institute Physician Postdoctoral Fellow during a portion of this work. P.O.B. is a Howard Hughes Medical Institute Associate Investigator. This work was supported by grants from the National Institutes of Health, the Howard Hughes Medical Institute, the Norwegian Cancer Society, and the Norwegian Research Council.

1. Kallioniemi, A., Kallioniemi, O. P., Sudar, D., Rutovitz, D., Gray, J. W., Waldman, F. & Pinkel, D. (1992) *Science* 258, 818–821.
2. Kallioniemi, A., Kallioniemi, O. P., Piper, J., Tanner, M., Stokke, T., Chen, L., Smith, H. S., Pinkel, D., Gray, J. W. & Waldman, F. M. (1994) *Proc. Natl. Acad. Sci. USA* 91, 2156–2160.
3. Tirkkonen, M., Tanner, M., Karhu, R., Kallioniemi, A., Isola, J. & Kallioniemi, O. P. (1998) *Genes Chromosomes Cancer* 21, 177–184.
4. Forozan, F., Mahlamaki, E. H., Monni, O., Chen, Y., Veldman, R., Jiang, Y., Gooden, G. C., Ethier, S. P., Kallioniemi, A. & Kallioniemi, O. P. (2000) *Cancer Res.* 60, 4519–4525.
5. Solinas-Toldo, S., Lampel, S., Stilgenbauer, S., Nickolenko, J., Benner, A., Dohner, H., Cremer, T. & Lichter, P. (1997) *Genes Chromosomes Cancer* 20, 399–407.
6. Pinkel, D., Segraves, R., Sudar, D., Clark, S., Poole, I., Kowbel, D., Collins, C., Kuo, W. L., Chen, C., Zhai, Y., *et al.* (1998) *Nat. Genet.* 20, 207–211.
7. Pollack, J. R., Perou, C. M., Alizadeh, A. A., Eisen, M. B., Pergamenschikov, A., Williams, C. F., Jeffrey, S. S., Botstein, D. & Brown, P. O. (1999) *Nat. Genet.* 23, 41–46.
8. Perou, C. M., Sorlie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., Rees, C. A., Pollack, J. R., Ross, D. T., Johnsen, H., Akslen, L. A., *et al.* (2000) *Nature (London)* 406, 747–752.
9. Sorlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisen, M. B., van de Rijn, M., Jeffrey, S. S., *et al.* (2001) *Proc. Natl. Acad. Sci. USA* 98, 10869–10874.
10. Schuler, G. D. (1997) *J. Mol. Med.* 75, 694–698.
11. Lander, E. S., Linton, L. M., Birren, B., Nusbaum, C., Zody, M. C., Baldwin, J., Devon, K., Dewar, K., Doyle, M., FitzHugh, W., *et al.* (2001) *Nature (London)* 409, 860–921.
12. Fejzo, M. S., Godfrey, T., Chen, C., Waldman, F. & Gray, J. W. (1998) *Genes Chromosomes Cancer* 22, 105–113.
13. Hughes, T. R., Roberts, C. J., Dai, H., Jones, A. R., Meyer, M. R., Slade, D., Burchard, J., Dow, S., Ward, T. R., Kidd, M. J., *et al.* (2000) *Nat. Genet.* 25, 333–337.
14. Phillips, J. L., Hayward, S. W., Wang, Y., Vasselli, J., Pavlovich, C., Padilla-Nash, H., Pezullo, J. R., Ghadimi, B. M., Grossfeld, G. D., Rivera, A., *et al.* (2001) *Cancer Res.* 61, 8143–8149.
15. Platzer, P., Upender, M. B., Wilson, K., Willis, J., Lutterbaugh, J., Nosrati, A., Willson, J. K., Mack, D., Ried, T. & Markowitz, S. (2002) *Cancer Res.* 62, 1134–1138.
16. Albertson, D. G., Ylstra, B., Segraves, R., Collins, C., Dairkee, S. H., Kowbel, D., Kuo, W. L., Gray, J. W. & Pinkel, D. (2000) *Nat. Genet.* 25, 144–146.
17. Li, R., Yerganian, G., Duesberg, P., Kraemer, A., Willer, A., Rausch, C. & Hehlmann, R. (1997) *Proc. Natl. Acad. Sci. USA* 94, 14506–14511.
18. Rasnick, D. & Duesberg, P. H. (1999) *Biochem. J.* 340, 621–630.

Review

Translation Initiation in Cancer: A Novel Target for Therapy[1]

Funda Meric[2] and Kelly K. Hunt

Department of Surgical Oncology, The University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030

Abstract

Translation initiation is regulated in response to nutrient availability and mitogenic stimulation and is coupled with cell cycle progression and cell growth. Several alterations in translational control occur in cancer. Variant mRNA sequences can alter the translational efficiency of individual mRNA molecules, which in turn play a role in cancer biology. Changes in the expression or availability of components of the translational machinery and in the activation of translation through signal transduction pathways can lead to more global changes, such as an increase in the overall rate of protein synthesis and translational activation of the mRNA molecules involved in cell growth and proliferation. We review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to help elucidate new therapeutic avenues.

Introduction

The fundamental principle of molecular therapeutics in cancer is to exploit the differences in gene expression between cancer cells and normal cells. With the advent of cDNA array technology, most efforts have concentrated on identifying differences in gene expression at the level of mRNA, which can be attributable either to DNA amplification or to differences in transcription. Gene expression is quite complicated, however, and is also regulated at the level of mRNA stability, mRNA translation, and protein stability.

The power of translational regulation has been best recognized among developmental biologists, because transcription does not occur in early embryogenesis in eukaryotes. For example, in *Xenopus*, the period of transcriptional quiescence continues until the embryo reaches midblastula transition, the 4000-cell stage. Therefore, all necessary mRNA molecules are transcribed during oogenesis and stockpiled in a translationally inactive, masked form. The mRNA are translationally activated at appropriate times during oocyte maturation, fertilization, and early embryogenesis and thus, are under strict translational control.

Translation has an established role in cell growth. Basically, an increase in protein synthesis occurs as a consequence of mitogenesis. Until recently, however, little was known about the alterations in mRNA translation in cancer, and much is yet to be discovered about their role in the development and progression of cancer. Here we review the basic principles of translational control, the alterations encountered in cancer, and selected therapies targeting translation initiation to elucidate potential new therapeutic avenues.

Basic Principles of Translational Control

Mechanism of Translation Initiation

Translation initiation is the main step in translational regulation. Translation initiation is a complex process in which the initiator tRNA and the 40S and 60S ribosomal subunits are recruited to the 5′ end of a mRNA molecule and assembled by eukaryotic translation initiation factors into an 80S ribosome at the start codon of the mRNA (Fig. 1). The 5′ end of eukaryotic mRNA is capped, *i.e.*, contains the cap structure m^7GpppN (7-methylguanosine-triphospho-5′-ribonucleoside). Most translation in eukaryotes occurs in a cap-dependent fashion, *i.e.*, the cap is specifically recognized by the eIF4E,[3] which binds the 5′ cap. The eIF4F translation initiation complex is then formed by the assembly of eIF4E, the RNA helicase eIF4A, and eIF4G, a scaffolding protein that mediates the binding of the 40S ribosomal subunit to the mRNA molecule through interaction with the eIF3 protein present on the 40S ribosome. eIF4A and eIF4B participate in melting the secondary structure of the 5′ UTR of the mRNA. The 43S initiation complex (40S/eIF2/Met-tRNA/GTP complex) scans the mRNA in a 5′→3′ direction until it encounters an AUG start codon. This start codon is then base-paired to the anticodon of initiator tRNA, forming the 48S initiation complex. The initiation factors are then displaced from the 48S complex, and the 60S ribosome joins to form the 80S ribosome.

Unlike most eukaryotic translation, translation initiation of certain mRNAs, such as the picornavirus RNA, is cap independent and occurs by internal ribosome entry. This mechanism does not require eIF4E. Either the 43S complex can bind the initiation codon directly through interaction with the IRES in the 5′ UTR such as in the encephalomyocarditis virus, or it can

Received 5/16/02; revised 7/12/02; accepted 7/22/02.
[1] F. M. is supported by The University of Texas M. D. Anderson Cancer Center Physician-Scientist Program and by NIH Grant 1KO8-CA 91895-01. K. K. H. is supported by Department of Defense Award DAMD-17-97-1-7162.
[2] To whom requests for reprints should be addressed, at Department of Surgical Oncology, Box 444, The University of Texas M. D. Anderson Cancer Center, 1515 Holcombe Boulevard, Houston, TX 77030. Phone: (713) 745-4453; Fax: (713) 745-4926; E-mail: fmeric@mdanderson.org.

[3] The abbreviations used are: eIF4E, eukaryotic initiation factor 4E; UTR, untranslated region; IRES, internal ribosome entry site; 4E-BP1, eukaryotic initiation factor 4E-binding protein 1; S6K, ribosomal p70 S6 kinase; mTOR, mammalian target of rapamycin; ATM, ataxia telangiectasia mutated; PI3K, phosphatidylinositol 3-kinase; PTEN, phosphatase and tensin homolog deleted from chromosome 10; PP2A, protein phosphatase 2A; TGF-β3, transforming growth factor-β3; PAP, poly(A) polymerase; EPA, eicosopentaenoic acid; *mda-7*, melanoma differentiation-associated gene 7.

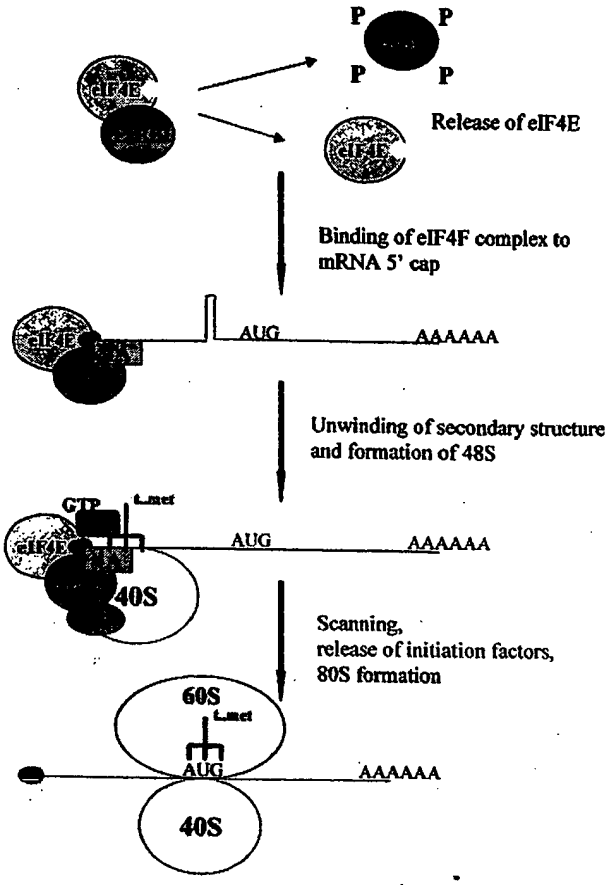


Fig. 1. Translation initiation in eukaryotes. The 4E-BPs are hyperphosphorylated to release eIF4E so that it can interact with the 5' cap, and the eIF4F initiation complex is assembled. The interaction of poly(A) binding protein with the initiation complex and circularization of the mRNA is not depicted in the diagram. The secondary structure of the 5' UTR is melted, the 40S ribosomal subunit is bound to eIF3, and the ternary complex consisting of eIF2, GTP, and the Met-tRNA are recruited to the mRNA. The ribosome scans the mRNA in a 5'→3' direction until an AUG start codon is found in the appropriate sequence context. The initiation factors are released, and the large ribosomal subunit is recruited.

initially attach to the IRES and then reach the initiation codon by scanning or transfer, as is the case with the poliovirus (1).

Regulation of Translation Initiation

Translation initiation can be regulated by alterations in the expression or phosphorylation status of the various factors involved. Key components in translational regulation that may provide potential therapeutic targets follow.

eIF4E. eIF4E plays a central role in translation regulation. It is the least abundant of the initiation factors and is considered the rate-limiting component for initiation of cap-dependent translation. eIF4E may also be involved in mRNA splicing, mRNA 3' processing, and mRNA nucleocytoplasmic transport (2). eIF4E expression can be increased at the transcriptional level in response to serum or growth factors (3). eIF4E overexpression may cause preferential translation of mRNAs containing excessive secondary structure in their 5' UTR that are normally discriminated against by the translational machinery and thus are inefficiently translated (4–7). As examples of this, overexpression of eIF4E promotes increased translation of vascular endothelial growth factor, fibroblast growth factor-2, and cyclin D1 (2, 8, 9).

Another mechanism of control is the regulation of eIF4E phosphorylation. eIF4E phosphorylation is mediated by the mitogen-activated protein kinase-interacting kinase 1, which is activated by the mitogen-activated pathway activating extracellular signal-related kinases and the stress-activated pathway acting through p38 mitogen-activated protein kinase (10–13). Several mitogens, such as serum, platelet-derived growth factor, epidermal growth factor, insulin, angiotensin II, src kinase overexpression, and ras overexpression, lead to eIF4E phosphorylation (14). The phosphorylation status of eIF4E is usually correlated with the translational rate and growth status of the cell; however; eIF4E phosphorylation has also been observed in response to some cellular stresses when translational rates actually decrease (15). Thus, further study is needed to understand the effects of eIF4E phosphorylation on eIF4E activity.

Another mechanism of regulation is the alteration of eIF4E availability by the binding of eIF4E to the eIF4E-binding proteins (4E-BP, also known as PHAS-I). 4E-BPs compete with eIF4G for a binding site in eIF4E. The binding of eIF4E to the best characterized eIF4E-binding protein, 4E-BP1, is regulated by 4E-BP1 phosphorylation. Hypophosphorylated 4E-BP1 binds to eIF4E, whereas 4E-BP1 hyperphosphorylation decreases this binding. Insulin, angiotensin, epidermal growth factor, platelet-derived growth factor, hepatocyte growth factor, nerve growth factor, insulin-like growth factors I and II, interleukin 3, granulocyte-macrophage colony-stimulating factor + steel factor, gastrin, and the adenovirus have all been reported to induce phosphorylation of 4E-BP1 and to decrease the ability of 4E-BP1 to bind eIF4E (15, 16). Conversely, deprivation of nutrients or growth factors results in 4E-BP1 dephosphorylation, an increase in eIF4E binding, and a decrease in cap-dependent translation.

p70 S6 Kinase. Phosphorylation of ribosomal 40S protein S6 by S6K is thought to play an important role in translational regulation. S6K −/− mouse embryonic cells proliferate more slowly than do parental cells, demonstrating that S6K has a positive influence on cell proliferation (17). S6K regulates the translation of a group of mRNAs possessing a 5' terminal oligopyrimidine tract (5' TOP) found at the 5' UTR of ribosomal protein mRNAs and other mRNAs coding for components of the translational machinery. Phosphorylation of S6K is regulated in part based on the availability of nutrients (18, 19) and is stimulated by several growth factors, such as platelet-derived growth factor and insulin-like growth factor I (20).

eIF2α Phosphorylation. The binding of the initiator tRNA to the small ribosomal unit is mediated by translation initiation factor eIF2. Phosphorylation of the α-subunit of eIF2 prevents formation of the eIF2/GTP/Met-tRNA complex and inhibits global protein synthesis (21, 22). eIF2α is phosphorylated under a variety of conditions, such as viral infection, nutrient deprivation, heme deprivation, and apoptosis (22). eIF2α is phosphorylated by heme-regulated inhibitor, nutrient-regulated protein kinase, and the IFN-induced, double-stranded RNA-activated protein kinase (PKR; Ref. 23).

The mTOR Signaling Pathway. The macrolide antibiotic rapamycin (Sirolimus; Wyeth-Ayerst Research, Collegeville, PA) has been the subject of intensive study because it inhibits signal transduction pathways involved in T-cell activation. The rapamycin-sensitive component of these pathways is mTOR (also called FRAP or RAFT1). mTOR is the mammalian homologue of the yeast TOR proteins that regulate G_1 progression and translation in response to nutrient availability (24). mTOR is a serine-threonine kinase that modulates translation initiation by altering the phosphorylation status of 4E-BP1 and S6K (Fig. 2; Ref. 25).

4E-BP1 is phosphorylated on multiple residues. mTOR phosphorylates the Thr-37 and Thr-46 residues of 4E-BP1 *in vitro* (26); however, phosphorylation at these sites is not associated with a loss of eIF4E binding. Phosphorylation of Thr-37 and Thr-46 is required for subsequent phosphorylation at several COOH-terminal, serum-sensitive sites; a combination of these phosphorylation events appears to be needed to inhibit the binding of 4E-BP1 to eIF4E (25). The product of the *ATM* gene, p38/MSK1 pathway, and protein kinase Cσ also play a role in 4E-BP1 phosphorylation (27–29).

S6K and 4E-BP1 are also regulated, in part, by PI3K and its downstream protein kinase Akt. PTEN is a phosphatase that negatively regulates PI3K signaling. PTEN null cells have constitutively active of Akt, with increased S6K activity and S6 phosphorylation (30). S6K activity is inhibited both by PI3K inhibitors wortmannin and LY294002 and by mTOR inhibitor rapamycin (24). Akt phosphorylates Ser-2448 in mTOR *in vitro*, and this site is phosphorylated upon Akt activation *in vivo* (31–33). Thus, mTOR is regulated by the PI3K/Akt pathway; however, this does not appear to be the only mode of regulation of mTOR activity. Whether the PI3K pathway also regulates S6K and 4E-BP1 phosphorylation independent of mTOR is controversial.

Interestingly, mTOR autophosphorylation is blocked by wortmannin but not by rapamycin (34). This seeming inconsistency suggests that mTOR-responsive regulation of 4E-BP1 and S6K activity occurs through a mechanism other than intrinsic mTOR kinase activity. An alternate pathway for 4E-BP1 and S6K phosphorylation by mTOR activity is by the inhibition of a phosphatase. Treatment with calyculin A, an inhibitor of phosphatases 1 and 2A, reduces rapamycin-induced dephosphorylation of 4E-BP1 and S6K by rapamycin (35). PP2A interacts with full-length S6K but not with a S6K mutant that is resistant to dephosphorylation resulting from rapamycin. mTOR phosphorylates PP2A *in vitro*; however, how this process alters PP2A activity is not known. These results are consistent with the model that phosphorylation of a phosphatase by mTOR prevents dephosphorylation of 4E-BP1 and S6K, and conversely, that nutrient deprivation and rapamycin block inhibition of the phosphatase by mTOR.

Polyadenylation. The poly(A) tail in eukaryotic mRNA is important in enhancing translation initiation and mRNA stability. Polyadenylation plays a key role in regulating gene expression during oogenesis and early embryogenesis. Some mRNA that are translationally inactive in the oocyte are polyadenylated concomitantly with translational activation in oocyte maturation, whereas other mRNAs that are translationally active during oogenesis are deadenylated and translationally silenced (36–38). Thus, control of poly(A) tail synthesis is an important regulatory step in gene expression. The 5′ cap and poly(A) tail are thought to function synergistically to regulate mRNA translational efficiency (39, 40).

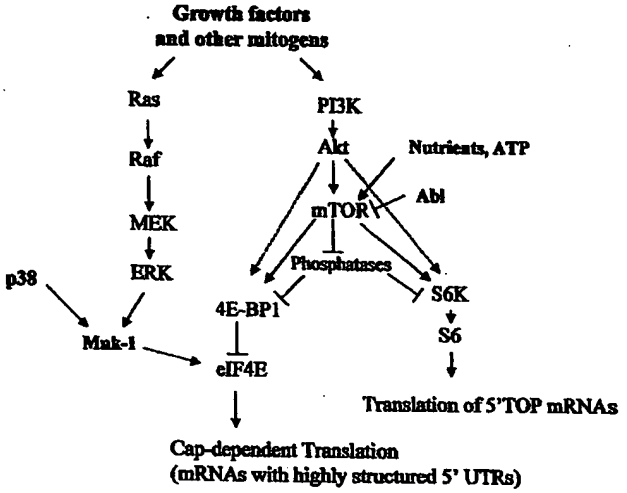


Fig. 2. Regulation of translation initiation by signal transduction pathways. Signaling via p38, extracellular signal-related kinase, PI3K, and mTOR can all activate translation initiation.

RNA Packaging. Most RNA-binding proteins are assembled on a transcript at the time of transcription, thus determining the translational fate of the transcript (41). A highly conserved family of Y-box proteins is found in cytoplasmic messenger ribonucleoprotein particles, where the proteins are thought to play a role in restricting the recruitment of mRNA to the translational machinery (41–43). The major mRNA-associated protein, YB-1, destabilizes the interaction of eIF4E and the 5′ mRNA cap *in vitro*, and overexpression of YB-1 results in translational repression *in vivo* (44). Thus, alterations in RNA packaging can also play an important role in translational regulation.

Translation Alterations Encountered in Cancer

Three main alterations at the translational level occur in cancer: variations in mRNA sequences that increase or decrease translational efficiency, changes in the expression or availability of components of the translational machinery, and activation of translation through aberrantly activated signal transduction pathways. The first alteration affects the translation of an individual mRNA that may play a role in carcinogenesis. The second and third alterations can lead to more global changes, such as an increase in the overall rate of protein synthesis, and the translational activation of several mRNA species.

Variations in mRNA Sequence

Variations in mRNA sequence affect the translational efficiency of the transcript. A brief description of these variations and examples of each mechanism follow.

Mutations. Mutations in the mRNA sequence, especially in the 5′ UTR, can alter its translational efficiency, as seen in the following examples.

c-myc. Salto *et al.* proposed that translation of full-length c-*myc* is repressed, whereas in several Burkitt lymphomas that have deletions of the mRNA 5′ UTR, translation of *c-myc* is more efficient (45). More recently, it was reported that the 5′ UTR of c-*myc* contains an IRES, and thus c-*myc* translation can be initiated by a cap-independent as well as a cap-dependent mechanism (46, 47). In patients with multiple myeloma, a C→T mutation in the c-myc IRES was identified (48) and found to cause an enhanced initiation of translation via internal ribosomal entry (49).

BRCA1. A somatic point mutation (117 G→C) in position −3 with respect to the start codon of the *BRCA1* gene was identified in a highly aggressive sporadic breast cancer (50). Chimeric constructs consisting of the wild-type or mutated *BRCA1* 5′ UTR and a downstream luciferase reporter demonstrated a decrease in the translational efficiency with the 5′ UTR mutation.

Cyclin-dependent Kinase Inhibitor 2A. Some inherited melanoma kindreds have a G→T transversion at base −34 of cyclin-dependent kinase inhibitor-2A, which encodes a cyclin-dependent kinase 4/cyclin-dependent kinase 6 kinase inhibitor important in G_1 checkpoint regulation (51). This mutation gives rise to a novel AUG translation initiation codon, creating an upstream open reading frame that competes for scanning ribosomes and decreases translation from the wild-type AUG.

Alternate Splicing and Alternate Transcription Start Sites. Alterations in splicing and alternate transcription sites can lead to variations in 5′ UTR sequence, length, and secondary structure, ultimately impacting translational efficiency.

ATM. The *ATM* gene has four noncoding exons in its 5′ UTR that undergo extensive alternative splicing (52). The contents of 12 different 5′ UTRs that show considerable diversity in length and sequence have been identified. These divergent 5′ leader sequences play an important role in the translational regulation of the *ATM* gene.

mdm. In a subset of tumors, overexpression of the oncoprotein mdm2 results in enhanced translation of the *mdm2* mRNA. Use of different promoters leads to two *mdm2* transcripts that differ only in their 5′ leaders (53). The longer 5′ UTR contains two upstream open reading frames, and this mRNA is loaded with ribosomes inefficiently compared with the short 5′ UTR.

BRCA1. In a normal mammary gland, *BRCA1* mRNA is expressed with a shorter leader sequence (5′UTRa), whereas in sporadic breast cancer tissue, *BRCA1* mRNA is expressed with a longer leader sequence (5′ UTRb); the translational efficiency of transcripts containing 5′ UTRb is 10 times lower than that of transcripts containing 5′ UTRa (54).

TGF-β3. *TGF-β3* mRNA includes a 1.1-kb 5′ UTR, which exerts an inhibitory effect on translation. Many human breast cancer cell lines contain a novel *TGF-β3* transcript with a 5′ UTR that is 870 nucleotides shorter and has a 7-fold greater translational efficiency than the normal *TGF-β3* mRNA (55).

Alternate Polyadenylation Sites. Multiple polyadenylation signals leading to the generation of several transcripts with differing 3′ UTR have been described for several mRNA species, such as the *RET* proto-oncogene (56), *ATM* gene (52), tissue inhibitor of metalloproteinases-3 (57), *RHOA* proto-oncogene (58), and calmodulin-I (59). Although the effect of these alternate 3′ UTRs on translation is not yet known, they may be important in RNA-protein interactions that affect translational recruitment. The role of these alterations in cancer development and progression is unknown.

Alterations in the Components of the Translation Machinery

Alterations in the components of translation machinery can take many forms.

Overexpresssion of eIF4E. Overexpression of eIF4E causes malignant transformation in rodent cells (60) and the deregulation of HeLa cell growth (61). Polunovsky *et al.* (62) found that eIF4E overexpression substitutes for serum and individual growth factors in preserving viability of fibroblasts, which suggests that eIF4E can mediate both proliferative and survival signaling.

Elevated levels of eIF4E mRNA have been found in a broad spectrum of transformed cell lines (63). eIF4E levels are elevated in all ductal carcinoma *in situ* specimens and invasive ductal carcinomas, compared with benign breast specimens evaluated with Western blot analysis (64, 65). Preliminary studies suggest that this overexpression is attributable to gene amplification (66).

There are accumulating data suggesting that eIF4E overexpression can be valuable as a prognostic marker. eIF4E overexpression was found in a retrospective study to be a marker of poor prognosis in stages I to III breast carcinoma (67). Verification of the prognostic value of eIF4E in breast cancer is now under way in a prospective trial (67). However, in a different study, eIF4E expression was correlated with the aggressive behavior of non-Hodgkin's lymphomas (68). In a prospective analysis of patients with head and neck cancer, elevated levels of eIF4E in histologically tumor-free surgical margins predicted a significantly increased risk of local-regional recurrence (9). These results all suggest that eIF4E overexpression can be used to select patients who might benefit from more aggressive systemic therapy. Furthermore, the head and neck cancer data suggest that eIF4E overexpression is a field defect and can be used to guide local therapy.

Alterations in Other Initiation Factors. Alterations in a number of other initiation factors have been associated with cancer. Overproduction of eIF4G, similar to eIF4E, leads to malignant transformation *in vitro* (69). eIF-2α is found in increased levels in bronchioloalveolar carcinomas of the lung (3). Initiation factor eIF-4A1 is overexpressed in melanoma (70) and hepatocellular carcinoma (71). The p40 subunit of translation initiation factor 3 is amplified and overexpressed in breast and prostate cancer (72), and the eIF3-p110 subunit is overexpressed in testicular seminoma (73). The role that overexpression of these initiation factors plays on the development and progression of cancer, if any, is not known.

Overexpression of S6K. S6K is amplified and highly overexpressed in the MCF7 breast cancer cell line, compared with normal mammary epithelium (74). In a study by Barlund *et al.* (74), S6K was amplified in 59 of 668 primary breast tumors, and a statistically significant association was observed between amplification and poor prognosis.

Overexpression of PAP. PAP catalyzes 3′ poly(A) synthesis. PAP is overexpressed in human cancer cells compared with normal and virally transformed cells (75). PAP enzymatic activity in breast tumors has been correlated with PAP protein levels (76) and, in mammary tumor cytosols, was found to be an independent factor for predicting survival (76). Little is known, however, about how PAP expression or activity affects the translational profile.

Alterations in RNA-binding Proteins. Even less is known about alterations in RNA packaging in cancer. Increased expression and nuclear localization of the RNA-binding protein YB-1 are indicators of a poor prognosis for breast cancer (77), non-small cell lung cancer (78), and ovarian cancer (79). However, this effect may be mediated at least in part at the level of transcription, because YB-1 increases chemoresistance by enhancing the transcription of a multidrug resistance gene (80).

Activation of Signal Transduction Pathways

Activation of signal transduction pathways by loss of tumor suppressor genes or overexpression of certain tyrosine kinases can contribute to the growth and aggressiveness of tumors. An important mutant in human cancers is the tumor suppressor gene *PTEN*, which leads to the activation of the PI3K/Akt pathway. Activation of PI3K and Akt induces the oncogenic transformation of chicken embryo fibroblasts. The transformed cells show constitutive phosphorylation of S6K and of 4E-BP1 (81). A mutant Akt that retains kinase activity but does not phosphorylate S6K or 4E-BP1 does not transform fibroblasts, which suggests a correlation between the oncogenicity of PI3K and Akt and the phosphorylation of S6K and 4E-BP1 (81).

Several tyrosine kinases such as platelet-derived growth factor, insulin-like growth factor, HER2/neu, and epidermal growth factor receptor are overexpressed in cancer. Because these kinases activate downstream signal transduction pathways known to alter translation initiation, activation of translation is likely to contribute to the growth and aggressiveness of these tumors. Furthermore, the mRNA for many of these kinases themselves are under translational control. For example, HER2/neu mRNA is translationally controlled both by a short upstream open reading frame that represses HER2/neu translation in a cell type-independent manner and by a distinct cell type-dependent mechanism that increases translational efficiency (82). HER2/neu translation is different in transformed and normal cells. Thus, it is possible that alterations at the translational level can in part account for the discrepancy between *HER2/neu* gene amplification detected by fluorescence *in situ* hybridization and protein levels detected by immunohistochemical assays.

Translation Targets of Selected Cancer Therapy

Components of the translation machinery and signal pathways involved in the activation of translation initiation represent good targets for cancer therapy.

Targeting the mTOR Signaling Pathway: Rapamycin and Tumstatin

Rapamycin inhibits the proliferation of lymphocytes. It was initially developed as an immunosuppressive drug for organ transplantation. Rapamycin with FKBP 12 (FK506-binding protein, M_r 12,000) binds to mTOR to inhibit its function.

Rapamycin causes a small but significant reduction in the initiation rate of protein synthesis (83). It blocks cell growth in part by blocking S6 phosphorylation and selectively suppressing the translation of 5′ TOP mRNAs, such as ribosomal proteins, and elongation factors (83–85). Rapamycin also blocks 4E-BP1 phosphorylation and inhibits cap-dependent but not cap-independent translation (17, 86).

The rapamycin-sensitive signal transduction pathway, activated during malignant transformation and cancer progression, is now being studied as a target for cancer therapy (87). Prostate, breast, small cell lung, glioblastoma, melanoma, and T-cell leukemia are among the cancer lines most sensitive to the rapamycin analogue CCI-779 (Wyeth-Ayerst Research; Ref. 87). In rhabdomyosarcoma cell lines, rapamycin is either cytostatic or cytocidal, depending on the p53 status of the cell; p53 wild-type cells treated with rapamycin arrest in the G_1 phase and maintain their viability, whereas p53 mutant cells accumulate in G_1 and undergo apoptosis (88, 89). In a recently reported study using human primitive neuroectodermal tumor and medulloblastoma models, rapamycin exhibited more cytotoxicity in combination with cisplatin and camptothecin than as a single agent. *In vivo*, CCI-779 delayed growth of xenografts by 160% after 1 week of therapy and 240% after 2 weeks. A single high-dose administration caused a 37% decrease in tumor volume. Growth inhibition *in vivo* was 1.3 times greater, with cisplatin in combination with CCI-779 than with cisplatin alone (90). Thus, preclinical studies suggest that rapamycin analogues are useful as single agents and in combination with chemotherapy.

Rapamycin analogues CCI-779 and RAD001 (Novartis, Basel, Switzerland) are now in clinical trials. Because of the known effect of rapamycin on lymphocyte proliferation, a potential problem with rapamycin analogues is immunosuppression. However, although prolonged immunosuppression can result from rapamycin and CCI-779 administered on continuous-dose schedules, the immunosuppressive effects of rapamycin analogues resolve in ~24 h after therapy (91). The principal toxicities of CCI-779 have included dermatological toxicity, myelosuppression, infection, mucositis, diarrhea, reversible elevations in liver function tests, hyperglycemia, hypokalemia, hypocalcemia, and depression (87, 92–94). Phase II trials of CCI-779 have been conducted in advanced renal cell carcinoma and in stage III/IV breast carcinoma patients who failed with prior chemotherapy. In the results reported in abstract form, although there were no complete responses, partial responses were documented in both renal cell carcinoma and in breast carcinoma (94, 95). Thus, CCI-779 has documented preliminary clinical activity in a previously treated, unselected patient population.

Active investigation is under way into patient selection for mTOR inhibitors. Several studies have found an enhanced efficacy of CCI-779 in PTEN-null tumors (30, 96). Another study found that six of eight breast cancer cell lines were responsive to CCI-779, although only two of these lines lacked PTEN (97) There was, however, a positive correlation between Akt activation and CCI-779 sensitivity (97). This correlation suggests that activation of the PI3K-Akt pathway,

regardless of whether it is attributable to a PTEN mutation or to overexpression of receptor tyrosine kinases, makes cancer cell amenable to mTOR-directed therapy. In contrast, lower levels of the target of mTOR, 4E-BP1, are associated with rapamycin resistance; thus, a lower 4E-BP1/eIF4E ratio may predict rapamycin resistance (98).

Another mode of activity for rapamycin and its analogues appears to be through inhibition of angiogenesis. This activity may be both through direct inhibition of endothelial cell proliferation as a result of mTOR inhibition in these cells or by inhibition of translation of such proangiogenic factors as vascular endothelial growth factor in tumor cells (99, 100).

The angiogenesis inhibitor tumstatin, another anticancer drug currently under study, was also found recently to inhibit translation in endothelial cells (101). Through a requisite interaction with integrin, tumstatin inhibits activation of the PI3K/Akt pathway and mTOR in endothelial cells and prevents dissociation of eIF4E from 4E-BP1, thereby inhibiting cap-dependent translation. These findings suggest that endothelial cells are especially sensitive to therapies targeting the mTOR-signaling pathway.

Targeting eIF2α: EPA, Clotrimazole, mda-7, and Flavonoids

EPA is an n-3 polyunsaturated fatty acid found in the fish-based diets of populations having a low incidence of cancer (102). EPA inhibits the proliferation of cancer cells (103), as well as in animal models (104, 105). It blocks cell division by inhibiting translation initiation (105). EPA releases Ca^{2+} from intracellular stores while inhibiting their refilling, thereby activating PKR. PKR, in turn phosphorylates and inhibits eIF2α, resulting in the inhibition of protein synthesis at the level of translation initiation. Similarly, clotrimazole, a potent antiproliferative agent *in vitro* and *in vivo*, inhibits cell growth through depletion of Ca^{2+} stores, activation of PKR, and phosphorylation of eIF2α (106). Consequently, clotrimazole preferentially decreases the expression of cyclins A, E, and D1, resulting in blockage of the cell cycle in G_1.

mda-7 is a novel tumor suppressor gene being developed as a gene therapy agent. Adenoviral transfer of *mda-7* (Ad-mda7) induces apoptosis in many cancer cells including breast, colorectal, and lung cancer (107–109). Ad-mda7 also induces and activates PKR, which leads to phosphorylation of eIF2α and induction of apoptosis (110).

Flavonoids such as genistein and quercetin suppress tumor cell growth. All three mammalian eIF2α kinases, PKR, heme-regulated inhibitor, and PERK/PEK, are activated by flavonoids, with phosphorylation of eIF2α and inhibition of protein synthesis (111).

Targeting eIF4A and eIF4E: Antisense RNA and Peptides

Antisense expression of eIF4A decreases the proliferation rate of melanoma cells (112). Sequestration of eIF4E by overexpression of 4E-BP1 is proapoptotic and decreases tumorigenicity (113, 114). Reduction of eIF4E with antisense RNA decreases soft agar growth, increases tumor latency, and increases the rates of tumor doubling times (7). Antisense eIF4E RNA treatment also reduces the expression of angiogenic factors (115) and has been proposed as a potential adjuvant therapy for head and neck cancers, particularly when elevated eIF4E is found in surgical margins. Small molecule inhibitors that bind the eIF4G/4E-BP1-binding domain of eIF4E are proapoptotic (116) and are also being actively pursued.

Exploiting Selective Translation for Gene Therapy

A different therapeutic approach that takes advantage of the enhanced cap-dependent translation in cancer cells is the use of gene therapy vectors encoding suicide genes with highly structured 5′ UTR. These mRNA would thus be at a competitive disadvantage in normal cells and not translate well, whereas in cancer cells, they would translate more efficiently. For example, the introduction of the 5′ UTR of fibroblast growth factor-2 5′ to the coding sequence of *herpes simplex virus type-1 thymidine kinase* gene, allows for selective translation of *herpes simplex virus type-1 thymidine kinase* gene in breast cancer cell lines compared with normal mammary cell lines and results in selective sensitivity to ganciclovir (117).

Toward the Future

Translation is a crucial process in every cell. However, several alterations in translational control occur in cancer. Cancer cells appear to need an aberrantly activated translational state for survival, thus allowing the targeting of translation initiation with surprisingly low toxicity. Components of the translational machinery, such as eIF4E, and signal transduction pathways involved in translation initiation, such mTOR, represent promising targets for cancer therapy. Inhibitors of the mTOR have already shown some preliminary activity in clinical trials. It is possible that with the development of better predictive markers and better patient selection, response rates to single-agent therapy can be improved. Similar to other cytostatic agents, however, mTOR inhibitors are most likely to achieve clinical utility in combination therapy. In the interim, our increasing understanding of translation initiation and signal transduction pathways promise to lead to the identification of new therapeutic targets in the near future.

Acknowledgments

We thank Gayle Nesom from The University of Texas M. D. Anderson Cancer Center Department of Scientific Publications for editorial assistance and Dr. Elmer Bernstam for assistance with manuscript preparation.

References

1. Pestova, T. V., Kolupaeva, V. G., Lomakin, I. B., Pilipenko, E. V., Shatsky, I. N., Agol, V. I., and Hellen, C. U. Molecular mechanisms of translation initiation in eukaryotes. Proc. Natl. Acad. Sci. USA, *98:* 7029–7036, 2001.

2. Rosenwald, I. B., Kaspar, R., Rousseau, D., Gehrke, L., Leboulch, P., Chen, J. J., Schmidt, E. V., Sonenberg, N., and London, I. M. Eukaryotic translation initiation factor 4E regulates expression of cyclin D1 at transcriptional and post-transcriptional levels. J. Biol. Chem., *270:* 21176–21180, 1995.

3. Rosenwald, I. B., Hutzler, M. J., Wang, S., Savas, L., and Fraire, A. E. Expression of eukaryotic translation initiation factors 4E and 2α is increased frequently in bronchioloalveolar but not in squamous cell carcinomas of the lung. Cancer (Phila.), *92:* 2164–2171, 2001.

4. Darveau, A., Pelletier, J., and Sonenberg, N. Differential efficiencies of *in vitro* translation of mouse c-myc transcripts differing in the 5′ untranslated region. Proc. Natl. Acad. Sci. USA, *82:* 2315–2319, 1985.

5. Kozak, M. Influences of mRNA secondary structure on initiation by eukaryotic ribosomes. Proc. Natl. Acad. Sci. USA, *83:* 2850–2854, 1986.

6. Koromilas, A. E., Lazaris-Karatzas, A., and Sonenberg, N. mRNAs containing extensive secondary structure in their 5′ non-coding region translate efficiently in cells overexpressing initiation factor eIF-4E. EMBO J., *11:* 4153–4158, 1992.

7. Rinker-Schaeffer, C. W., Graff, J. R., De Benedetti, A., Zimmer, S. G., and Rhoads, R. E. Decreasing the level of translation initiation factor 4E with antisense RNA causes reversal of ras-mediated transformation and tumorigenesis of cloned rat embryo fibroblasts. Int. J. Cancer, *55:* 841–847, 1993.

8. Kevil, C. G., De Benedetti, A., Payne, D. K., Coe, L. L., Laroux, F. S., and Alexander, J. S. Translational regulation of vascular permeability factor by eukaryotic initiation factor 4E: implications for tumor angiogenesis. Int. J. Cancer, *65:* 785–790, 1996.

9. Nathan, C. A., Franklin, S., Abreo, F. W., Nassar, R., De Benedetti, A., and Glass, J. Analysis of surgical margins with the molecular marker eIF4E: a prognostic factor in patients with head and neck cancer. J. Clin. Oncol., *17:* 2909–2914, 1999.

10. Fukunaga, R., and Hunter, T. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J., *16:* 1921–1933, 1997.

11. Waskiewicz, A. J., Flynn, A., Proud, C. G., and Cooper, J. A. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J., *16:* 1909–1920, 1997.

12. Wang, X., Flynn, A., Waskiewicz, A. J., Webb, B. L., Vries, R. G., Baines, I. A., Cooper, J. A., and Proud, C. G. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. J. Biol. Chem., *273:* 9373–9377, 1998.

13. Pyronnet, S., Imataka, H., Gingras, A. C., Fukunaga, R., Hunter, T., and Sonenberg, N. Human eukaryotic translation initiation factor 4G (eIF4G) recruits Mnk1 to phosphorylate eIF4E. EMBO J., *18:* 270–279, 1999.

14. Kleijn, M., Scheper, G. C., Voorma, H. O., and Thomas, A. A. Regulation of translation initiation factors by signal transduction. Eur. J. Biochem., *253:* 531–544, 1998.

15. Raught, B., and Gingras, A. C. eIF4E activity is regulated at multiple levels. Int. J. Biochem. Cell Biol., *31:* 43–57, 1999.

16. Takeuchi, K., Shibamoto, S., Nagamine, K., Shigemori, I., Omura, S., Kitamura, N., and Ito, F. Signaling pathways leading to transcription and translation cooperatively regulate the transient increase in expression of c-Fos protein. J. Biol. Chem., *276:* 26077–26083, 2001.

17. Kawasome, H., Papst, P., Webb, S., Keller, G. M., Johnson, G. L., Gelfand, E. W., and Terada, N. Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity. Proc. Natl. Acad. Sci. USA, *95:* 5033–5038, 1998.

18. Christie, G. R., Hajduch, E., Hundal, H. S., Proud, C. G., and Taylor, P. M. Intracellular sensing of amino acids in *Xenopus laevis* oocytes stimulates p70 S6 kinase in a target of rapamycin-dependent manner. J. Biol. Chem., *277:* 9952–9957, 2002.

19. Hara, K., Yonezawa, K., Weng, Q. P., Kozlowski, M. T., Belham, C., and Avruch, J. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J. Biol. Chem., *273:* 14484–14494, 1998.

20. Graves, L. M., Bornfeldt, K. E., Argast, G. M., Krebs, E. G., Kong, X., Lin, T. A., and Lawrence, J. C., Jr. cAMP- and rapamycin-sensitive regulation of the association of eukaryotic initiation factor 4E and the translational regulator PHAS-I in aortic smooth muscle cells. Proc. Natl. Acad. Sci. USA, *92:* 7222–7226, 1995.

21. Merrick, W. C., and Hershey, J. W. B. The pathway and mechanism of eukaryotic protein synthesis. *In:* J. W. B. Hershey and M. B. Mathews (eds.), Translational Control, pp. 31–69. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory, 1996.

22. Kimball, S. R. Eukaryotic initiation factor eIF2. Int. J. Biochem. Cell Biol., *31:* 25–29, 1999.

23. Jagus, R., Joshi, B., and Barber, G. N. PKR, apoptosis and cancer. Int. J. Biochem. Cell Biol., *31:* 123–138, 1999.

24. Thomas, G., and Hall, M. N. TOR signalling and control of cell growth. Curr. Opin. Cell Biol., *9:* 782–787, 1997.

25. Gingras, A. C., Raught, B., and Sonenberg, N. Regulation of translation initiation by FRAP/mTOR. Genes Dev., *15:* 807–826, 2001.

26. Gingras, A. C., Gygi, S. P., Raught, B., Polakiewicz, R. D., Abraham, R. T., Hoekstra, M. F., Aebersold, R., and Sonenberg, N. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev., *13:* 1422–1437, 1999.

27. Kumar, V., Pandey, P., Sabatini, D., Kumar, M., Majumder, P. K., Bharti, A., Carmichael, G., Kufe, D., and Karbhanda, S. Functional interaction between RAFT1/FRAP/mTOR and protein kinase Cδ in the regulation of cap-dependent initiation of translation. EMBO J., *19:* 1087–1097, 2000.

28. Yang, D. Q., and Kastan, M. B. Participation of ATM in insulin signalling through phosphorylation of eIF-4E-binding protein 1. Nat. Cell. Biol., *2:* 893–898, 2000.

29. Liu, G., Zhang, Y., Bode, A. M., Ma, W. Y., and Dong, Z. Phosphorylation of 4E-BP1 is mediated by the p38/MSK1 pathway in response to UVB irradiation. J. Biol. Chem., *277:* 8810–8816, 2002.

30. Neshat, M. S., Mellinghoff, I. K., Tran, C., Stiles, B., Thomas, G., Petersen, R., Frost, P., Gibbons, J. J., Wu, H., and Sawyers, C. L. Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR. Proc. Natl. Acad. Sci. USA, *98:* 10314–10319, 2001.

31. Sekulic, A., Hudson, C. C., Homme, J. L., Yin, P., Otterness, D. M., Karnitz, L. M., and Abraham, R. T. A direct linkage between the phosphoinositide 3-kinase-AKT signaling pathway and the mammalian target of rapamycin in mitogen-stimulated and transformed cells. Cancer Res., *60:* 3504–3513, 2000.

32. Scott, P. H., and Lawrence, J. C., Jr. Attenuation of mammalian target of rapamycin activity by increased cAMP in 3T3-L1 adipocytes. J. Biol. Chem., *273:* 34496–34501, 1998.

33. Reynolds, I. T., Bodine, S. C., and Lawrence, J. C., Jr. Control of Ser2448 phosphorylation in the mammalian target of rapamycin by insulin and skeletal muscle load. J. Biol. Chem., *277:* 17657–17662, 2002.

34. Peterson, R. T., Beal, P. A., Comb, M. J., and Schreiber, S. L. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. J. Biol. Chem., *275:* 7416–7423, 2000.

35. Peterson, R. T., Desai, B. N., Hardwick, J. S., and Schreiber, S. L. Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12-rapamycin-associated protein. Proc. Natl. Acad. Sci. USA, *96:* 4438–4442, 1999.

36. McGrew, L. L., Dworkin-Rastl, E., Dworkin, M. B., and Richter, J. D. Poly(A) elongation during *Xenopus* oocyte maturation is required for translational recruitment and is mediated by a short sequence element. Genes Dev., *3:* 803–815, 1989.

37. Sheets, M. D., Wu, M., and Wickens, M. Polyadenylation of c-mos mRNA as a control point in *Xenopus* meiotic maturation. Nature (Lond.), *374:* 511–516, 1995.

38. Varnum, S. M., and Wormington, W. M. Deadenylation of maternal mRNAs during *Xenopus* oocyte maturation does not require specific *cis*-sequences: a default mechanism for translational control. Genes Dev., *4:* 2278–2286, 1990.

39. Gallie, D. R. The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev., *5:* 2108–2116, 1991.

40. Sachs, A. B., and Varani, G. Eukaryotic translation initiation: there are (at least) two sides to every story. Nat. Struct. Biol., *7:* 356–361, 2000.

41. Wolffe, A. P., and Meric, F. Coupling transcription to translation: a novel site for the regulation of eukaryotic gene expression. Int. J. Biochem. Cell Biol., *28:* 247–257, 1996.

42. Evdokimova, V. M., Wei, C. L., Sitikov, A. S., Simonenko, P. N., Lazarev, O. A., Vasilenko, K. S., Ustinov, V. A., Hershey, J. W., and Ovchinnikov, L. P. The major protein of messenger ribonucleoprotein particles in somatic cells is a member of the Y-box binding transcription factor family. J. Biol. Chem., *270:* 3186–3192, 1995.

43. Matsumoto, K., Meric, F., and Wolffe, A. P. Translational repression dependent on the interaction of the *Xenopus* Y-box protein FRGY2 with mRNA. Role of the cold shock domain, tail domain, and selective RNA sequence recognition. J. Biol. Chem., *271:* 22706–22712, 1996.

44. Evdokimova, V., Ruzanov, P., Imataka, H., Raught, B., Svitkin, Y., Ovchinnikov, L. P., and Sonenberg, N. The major mRNA-associated protein YB-1 is a potent 5′ cap-dependent mRNA stabilizer. EMBO J., *20:* 5491–5502, 2001.

45. Saito, H., Hayday, A. C., Wiman, K., Hayward, W. S., and Tonegawa, S. Activation of the c-*myc* gene by translocation: a model for translational control. Proc. Natl. Acad. Sci. USA, *80:* 7476–7480, 1983.

46. Nanbru, C., Lafon, I., Audigier, S., Gensac, M. C., Vagner, S., Huez, G., and Prats, A. C. Alternative translation of the proto-oncogene c-*myc* by an internal ribosome entry site. J. Biol. Chem., *272:* 32061–32066, 1997.

47. Stoneley, M., Paulin, F. E., Le Quesne, J. P., Chappell, S. A., and Willis, A. E. c-Myc 5′ untranslated region contains an internal ribosome entry segment. Oncogene, *16:* 423–428, 1998.

48. Paulin, F. E., West, M. J., Sullivan, N. F., Whitney, R. L., Lyne, L., and Willis, A. E. Aberrant translational control of the c-*myc* gene in multiple myeloma. Oncogene, *13:* 505–513, 1996.

49. Chappell, S. A., LeQuesne, J. P., Paulin, F. E., de Schoolmeester, M. L., Stoneley, M., Soutar, R. L., Ralston, S. H., Helfrich, M. H., and Willis, A. E. A mutation in the c-myc-IRES leads to enhanced internal ribosome entry in multiple myeloma: a novel mechanism of oncogene de-regulation. Oncogene, *19:* 4437–4440, 2000.

50. Signori, E., Bagni, C., Papa, S., Primerano, B., Rinaldi, M., Amaldi, F., and Fazio, V. M. A somatic mutation in the 5′UTR of *BRCA1* gene in sporadic breast cancer causes down-modulation of translation efficiency. Oncogene, *20:* 4596–4600, 2001.

51. Liu, L., Dilworth, D., Gao, L., Monzon, J., Summers, A., Lassam, N., and Hogg, D. Mutation of the CDKN2A 5′ UTR creates an aberrant initiation codon and predisposes to melanoma. Nat. Genet., *21:* 128–132, 1999.

52. Savitsky, K., Platzer, M., Uziel, T., Gilad, S., Sartiel, A., Rosenthal, A., Elroy-Stein, O., Shiloh, Y., and Rotman, G. Ataxia-telangiectasia: structural diversity of untranslated sequences suggests complex post-transcriptional regulation of *ATM* gene expression. Nucleic Acids Res., *25:* 1678–1684, 1997.

53. Brown, C. Y., Mize, G. J., Pineda, M., George, D. L., and Morris, D. R. Role of two upstream open reading frames in the translational control of oncogene *mdm2*. Oncogene, *18:* 5631–5637, 1999.

54. Sobczak, K., and Krzyzosiak, W. J. Structural determinants of BRCA1 translational regulation. J. Biol. Chem., *277:* 17349–17358, 2002.

55. Arrick, B. A., Grendell, R. L., and Griffin, L. A. Enhanced translational efficiency of a novel transforming growth factor β3 mRNA in human breast cancer cells. Mol. Cell. Biol., *14:* 619–628, 1994.

56. Myers, S. M., Eng, C., Ponder, B. A., and Mulligan, L. M. Characterization of *RET* proto-oncogene 3′ splicing variants and polyadenylation sites: a novel C-terminus for RET. Oncogene, *11:* 2039–2045, 1995.

57. Byrne, J. A., Tomasetto, C., Rouyer, N., Bellocq, J. P., Rio, M. C., and Basset, P. The tissue inhibitor of *metalloproteinases-3* gene in breast carcinoma: identification of multiple polyadenylation sites and a stromal pattern of expression. Mol. Med., *1:* 418–427, 1995.

58. Moscow, J. A., He, R., Gudas, J. M., and Cowan, K. H. Utilization of multiple polyadenylation signals in the human *RHOA* protooncogene. Gene (Amst.), *144:* 229–236, 1994.

59. Senterre-Lesenfants, S., Alag, A. S., and Sobel, M. E. Multiple mRNA species are generated by alternate polyadenylation from the human *calmodulin-I* gene. J. Cell. Biochem., *58:* 445–454, 1995.

60. Lazaris-Karatzas, A., Montine, K. S., and Sonenberg, N. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5′ cap. Nature (Lond.), *345:* 544–547, 1990.

61. De Benedetti, A., and Rhoads, R. E. Overexpression of eukaryotic protein synthesis initiation factor 4E in HeLa cells results in aberrant growth and morphology. Proc. Natl. Acad. Sci. USA, *87:* 8212–8216, 1990.

62. Polunovsky, V. A., Rosenwald, I. B., Tan, A. T., White, J., Chiang, L., Sonenberg, N., and Bitterman, P. B. Translational control of programmed cell death: eukaryotic translation initiation factor 4E blocks apoptosis in growth-factor-restricted fibroblasts with physiologically expressed or deregulated Myc. Mol. Cell. Biol., *16:* 6573–6581, 1996.

63. Miyagi, Y., Sugiyama, A., Asai, A., Okazaki, T., Kuchino, Y., and Kerr, S. J. Elevated levels of eukaryotic translation initiation factor eIF-4E, mRNA in a broad spectrum of transformed cell lines. Cancer Lett., *91:* 247–252, 1995.

64. Kerekatte, V., Smiley, K., Hu, B., Smith, A., Gelder, F., and De Benedetti, A. The proto-oncogene/translation factor *eIF4E*: a survey of its expression in breast carcinomas. Int. J. Cancer, *64:* 27–31, 1995.

65. Li, B. D., Liu, L., Dawson, M., and De Benedetti, A. Overexpression of eukaryotic initiation factor 4E (eIF4E) in breast carcinoma. Cancer (Phila.), *79:* 2385–2390, 1997.

66. Sorrells, D. L., Black, D. R., Meschonat, C., Rhoads, R., De Benedetti, A., Gao, M., Williams, B. J., and Li, B. D. Detection of *eIF4E* gene amplification in breast cancer by competitive PCR. Ann. Surg. Oncol., *5:* 232–237, 1998.

67. Li, B. D., McDonald, J. C., Nassar, R., and De Benedetti, A. Clinical outcome in stage I to III breast carcinoma and eIF4E overexpression. Ann. Surg., *227:* 756–761; discussion, 761–763, 1998.

68. Wang, S., Rosenwald, I. B., Hutzler, M. J., Pihan, G. A., Savas, L., Chen, J. J., and Woda, B. A. Expression of the eukaryotic translation initiation factors 4E and 2α in non-Hodgkin's lymphomas. Am. J. Pathol., *155:* 247–255, 1999.

69. Fukuchi-Shimogori, T., Ishii, I., Kashiwagi, K., Mashiba, H., Ekimoto, H., and Igarashi, K. Malignant transformation by overproduction of translation initiation factor eIF4G. Cancer Res., *57:* 5041–5044, 1997.

70. Eberle, J., Krasagakis, K., and Orfanos, C. E. Translation initiation factor eIF-4A1 mRNA is consistently overexpressed in human melanoma cells *in vitro*. Int. J. Cancer, *71:* 396–401, 1997.

71. Shuda, M., Kondoh, N., Tanaka, K., Ryo, A., Wakatsuki, T., Hada, A., Goseki, N., Igari, T., Hatsuse, K., Aihara, T., Horiuchi, S., Shichita, M., Yamamoto, N., and Yamamoto, M. Enhanced expression of translation factor mRNAs in hepatocellular carcinoma. Anticancer Res., *20:* 2489–2494, 2000.

72. Nupponen, N. N., Porkka, K., Kakkola, L., Tanner, M., Persson, K., Borg, A., Isola, J., and Visakorpi, T. Amplification and overexpression of p40 subunit of eukaryotic translation initiation factor 3 in breast and prostate cancer. Am. J. Pathol., *154:* 1777–1783, 1999.

73. Rothe, M., Ko, Y., Albers, P., and Wernert, N. Eukaryotic initiation factor 3 p110 mRNA is overexpressed in testicular seminomas. Am. J. Pathol., *157:* 1597–1604, 2000.

74. Barlund, M., Forozan, F., Kononen, J., Bubendorf, L., Chen, Y., Bittner, M. L., Torhorst, J., Haas, P., Bucher, C., Sauter, G., Kallioniemi, O. P., and Kallioniemi, A. Detecting activation of ribosomal protein S6 kinase by complementary DNA and tissue microarray analysis. J. Natl. Cancer Inst. (Bethesda), *92:* 1252–1259, 2000.

75. Topalian, S. L., Kaneko, S., Gonzales, M. I., Bond, G. L., Ward, Y., and Manley, J. L. Identification and functional characterization of neo-poly(A) polymerase, an RNA processing enzyme overexpressed in human tumors. Mol. Cell. Biol., *21:* 5614–5623, 2001.

76. Scorilas, A., Talieri, M., Ardavanis, A., Courtis, N., Dimitriadis, E., Yotis, J., Tsiapalis, C. M., and Trangas, T. Polyadenylate polymerase enzymatic activity in mammary tumor cytosols: a new independent prognostic marker in primary breast cancer. Cancer Res., *60:* 5427–5433, 2000.

77. Janz, M., Harbeck, N., Dettmar, P., Berger, U., Schmidt, A., Jurchott, K., Schmitt, M., and Royer, H. D. Y-box factor YB-1 predicts drug resistance and patient outcome in breast cancer independent of clinically relevant tumor biologic factors HER2, uPA and PAI-1. Int. J. Cancer, *97:* 278–282, 2002.

78. Shibahara, K., Sugio, K., Osaki, T., Uchiumi, T., Maehara, Y., Kohno, K., Yasumoto, K., Sugimachi, K., and Kuwano, M. Nuclear expression of the Y-box binding protein, YB-1, as a novel marker of disease progression in non-small cell lung cancer. Clin. Cancer Res., *7:* 3151–3155, 2001.

79. Kamura, T., Yahata, H., Amada, S., Ogawa, S., Sonoda, T., Kobayashi, H., Mitsumoto, M., Kohno, K., Kuwano, M., and Nakano, H. Is nuclear expression of Y box-binding protein-1 a new prognostic factor in ovarian serous adenocarcinoma? Cancer (Phila.), *85:* 2450–2454, 1999.

80. Bargou, R. C., Jurchott, K., Wagener, C., Bergmann, S., Metzner, S., Bommert, K., Mapara, M. Y., Winzer, K. J., Dietel, M., Dorken, B., and Royer, H. D. Nuclear localization and increased levels of transcription factor YB-1 in primary human breast cancers are associated with intrinsic *MDR1* gene expression. Nat. Med., *3:* 447–450, 1997.

81. Aoki, M., Blazek, E., and Vogt, P. K. A role of the kinase mTOR in cellular transformation induced by the oncoproteins P3k and Akt. Proc. Natl. Acad. Sci. USA, *98:* 136–141, 2001.

82. Child, S. J., Miller, M. K., and Geballe, A. P. Cell type-dependent and -independent control of HER-2/neu translation. Int. J. Biochem. Cell Biol., *31:* 201–213, 1999.

83. Jefferies, H. B., Reinhard, C., Kozma, S. C., and Thomas, G. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc. Natl. Acad. Sci. USA, *91:* 4441–4445, 1994.

84. Terada, N., Patel, H. R., Takase, K., Kohno, K., Nairn, A. C., and Gelfand, E. W. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc. Natl. Acad. Sci. USA, *91:* 11477–11481, 1994.

85. Jefferies, H. B., Fumagalli, S., Dennis, P. B., Reinhard, C., Pearson, R. B., and Thomas, G. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. EMBO J., *16:* 3693–3704, 1997.

86. Beretta, L., Gingras, A. C., Svitkin, Y. V., Hall, M. N., and Sonenberg, N. Rapamycin blocks the phosphorylation of 4E-BP1 and inhibits cap-dependent initiation of translation. EMBO J., *15:* 658–664, 1996.

87. Hidalgo, M., and Rowinsky, E. K. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene, *19:* 6680–6686, 2000.

88. Hosoi, H., Dilling, M. B., Shikata, T., Liu, L. N., Shu, L., Ashmun, R. A., Germain, G. S., Abraham, R. T., and Houghton, P. J. Rapamycin causes poorly reversible inhibition of mTOR and induces p53-independent apoptosis in human rhabdomyosarcoma cells. Cancer Res., *59:* 886–894, 1999.

89. Huang, S., and Houghton, P. J. Resistance to rapamycin: a novel anticancer drug. Cancer Metastasis Rev., *20:* 69–78, 2001.

90. Geoerger, B., Kerr, K., Tang, C. B., Fung, K. M., Powell, B., Sutton, L. N., Phillips, P. C., and Janss, A. J. Antitumor activity of the rapamycin analog CCI-779 in human primitive neuroectodermal tumor/medulloblastoma models as single agent and in combination chemotherapy. Cancer Res., *61:* 1527–1532, 2001.

91. Gibbons, J. J., Discafani, C., Peterson, R., Hernandez, R., Skotnicki, J., and Frost, P. The effect of CCI-779, a novel macrolide anti-tumor agent, on the growth of human tumor cells *in vitro* and in nude mouse xenografts *in vivo*. Proc. Am. Assoc. Cancer Res., *40:* 301, 1999.

92. Hidalgo, M., Rowinsky, E., Erlichman, C., Marshall, B., Marks, R., Edwards, T., and Buckner, J. J. A Phase I and pharmacological study of CCI-779 cycle inhibitor. Ann. Oncol., *11* (Suppl. 4)*:* 133, 2001.

93. Alexandre, J., Raymond, E., Depenbrock, H., Mekhaldi, S., Angevin, E., Paillet, C., Hanauske, A., Frisch, J., Feussner, A., and Armand, J. P. CCI-779, a new rapamycin analog, has antitumor activity at doses inducing only mild cutaneous effects and mucositis: early results of an ongoing Phase I study. Proceedings of the 1999 AACR-NCI-EORTC International Conference, Clin. Cancer Res., *5* (Suppl.): 3730s, 1999.

94. Chan, S., Johnston, S., Scheulen, M. E., Mross, K., Morant, A., Lahr, A., Feussner, A., Berger, M., and Kirsch, T. First report: a Phase 2 study of the safety and activity of CCI-779 for patients with locally advanced or metastatic breast cancer failing prior chemotherapy. Proc. Am. Soc. Clin. Oncol., *21:* 44a, 2002.

95. Atkins, M. B., Hidalgo, M., Stadler, W., Logan, T., Dutcher, J. P., Hudes, G., Park, Y., Marshall, B., Boni, J., and Dukart, G. A randomized double-blind Phase 2 study of intravenous CCI-779 administered weekly to patients with advanced renal cell carcinoma. Proc. Am. Soc. Clin. Oncol., *21:* 10a, 2002.

96. Smith, S. G., Trinh, C. M., Inge, L. J., Thomas, G., Cloughsey, T. F., Sawyers, C. L., and Mischel, P. S. PTEN expression status predicts glioblastoma cell sensitivity to CCI-779. Proc. Am. Assoc. Cancer Res., *43:* 335, 2002.

97. Yu, K., Toral-Barza, L., Discafani, C., Zhang, W. G., Skotnicki, J., Frost, P., and Gibbons, J. J. mTOR, a novel target in breast cancer: the effect of CCI-779, an mTOR inhibitor, in preclinical models of breast cancer. Endocr. Relat. Cancer, *8:* 249–258, 2001.

98. Dilling, M. B., Germain, G. S., Dudkin, L., Jayaraman, A. L., Zhang, X., Harwood, F. C., and Houghton, P. J. 4E-binding proteins, the suppressors of eukaryotic initiation factor 4E, are downregulated in cells with acquired or intrinsic resistance to rapamycin. J. Biol. Chem., *277:* 13907–13917, 2002.

99. Guba, M., von Breitenbuch, P., Steinbauer, M., Koehl, G., Flegel, S., Hornung, M., Bruns, C. J., Zuelke, C., Farkas, S., Anthuber, M., Jauch, K. W., and Geissler, E. K. Rapamycin inhibits primary and metastatic tumor growth by antiangiogenesis: involvement of vascular endothelial growth factor. Nat. Med., *8:* 128–135, 2002.

100. Lane, H. A., Schell, C., Theuer, A., O'Reilly, T., and Wood, J. Antiangiogenic activity of RAD001, an orally active anticancer agent. Proc. Am. Assoc. Cancer Res., *43:* 184, 2002.

101. Maeshima, Y., Sudhakar, A., Lively, J. C., Ueki, K., Kharbanda, S., Kahn, C. R., Sonenberg, N., Hynes, R. O., and Kalluri, R. Tumstatin, an endothelial cell-specific inhibitor of protein synthesis. Science (Wash. DC), *295:* 140–143, 2002.

102. Caygill, C. P., Charlett, A., and Hill, M. J. Fat, fish, fish oil and cancer. Br. J. Cancer, *74:* 159–164, 1996.

103. Falconer, J. S., Ross, J. A., Fearon, K. C., Hawkins, R. A., O'Riordain, M. G., and Carter, D. C. Effect of eicosapentaenoic acid and other fatty acids on the growth *in vitro* of human pancreatic cancer cell lines. Br. J. Cancer, *69:* 826–832, 1994.

104. Noguchi, M., Minami, M., Yagasaki, R., Kinoshita, K., Earashi, M., Kitagawa, H., Taniya, T., and Miyazaki, I. Chemoprevention of DMBA-induced mammary carcinogenesis in rats by low-dose EPA and DHA. Br. J. Cancer, *75:* 348–353, 1997.

105. Palakurthi, S. S., Fluckiger, R., Aktas, H., Changolkar, A. K., Shahsafael, A., Harnett, S., Kilic, E., and Halperin, J. A. Inhibition of translation initiation mediates the anticancer effect of the n-3 polyunsaturated fatty acid eicosapentaenoic acid. Cancer Res., *60:* 2919–2925, 2000.

106. Aktas, H., Fluckiger, R., Acosta, J. A., Savage, J. M., Palakurthi, S. S., and Halperin, J. A. Depletion of intracellular Ca^{2+} stores, phosphorylation of eIF2α, and sustained inhibition of translation initiation mediate the anticancer effects of clotrimazole. Proc. Natl. Acad. Sci. USA, *95:* 8280–8285, 1998.

107. Mhashilkar, A. M., Schrock, R. D., Hindi, M., Liao, J., Sieger, K., Kourouma, F., Zou-Yang, X. H., Onishi, E., Takh, O., Vedvick, T. S., Fanger, G., Stewart, L., Watson, G. J., Snary, D., Fisher, P. B., Saeki, T., Roth, J. A., Ramesh, R., and Chada, S. Melanoma differentiation associated gene-7 (*mda-7*): a novel anti-tumor gene for cancer gene therapy. Mol. Med., *7:* 271–282, 2001.

108. Su, Z. Z., Madireddi, M. T., Lin, J. J., Young, C. S., Kitada, S., Reed, J. C., Goldstein, N. I., and Fisher, P. B. The cancer growth suppressor gene *mda-7* selectively induces apoptosis in human breast cancer cells and inhibits tumor growth in nude mice. Proc. Natl. Acad. Sci. USA, *95:* 14400–14405, 1998.

109. Saeki, T., Mhashilkar, A., Chada, S., Branch, C., Roth, J. A., and Ramesh, R. Tumor-suppressive effects by adenovirus-mediated *mda-7* gene transfer in non-small cell lung cancer cell *in vitro*. Gene Ther., *7:* 2051–2057, 2000.

110. Pataer, A., Vorburger, S. A., Barber, G. N., Chada, S., Mhashilkar, A. M., Zou-Yang, H., Stewart, A. L., Balachandran, S., Roth, J. A., Hunt, K. K., and Swisher, S. G. Adenoviral transfer of the melanoma differentiation-associated gene 7 (*mda7*) induces apoptosis of lung cancer cells via up-regulation of the double-stranded RNA-dependent protein kinase (PKR). Cancer Res., *62:* 2239–2243, 2002.

111. Ito, T., Warnken, S. P., and May, W. S. Protein synthesis inhibition by flavonoids: roles of eukaryotic initiation factor 2α kinases. Biochem. Biophys. Res. Commun., *265:* 589–594, 1999.

112. Eberle, J., Fecker, L. F., Bittner, J. U., Orfanos, C. E., and Geilen, C. C. Decreased proliferation of human melanoma cell lines caused by antisense RNA against translation factor eIF-4A1. Br. J. Cancer, *86:* 1957–1962, 2002.

113. Polunovsky, V. A., Gingras, A. C., Sonenberg, N., Peterson, M., Tan, A., Rubins, J. B., Manivel, J. C., and Bitterman, P. B. Translational control of the antiapoptotic function of Ras. J. Biol. Chem., *275:* 24776–24780, 2000.

114. D'Cunha, J., Kratzke, M. G., Alter, M. D., Polunovsky, V. A., Bitterman, P. B., and Kratzke, R. A. Over-expression of the translational repressor 4E-BP1 inhibits NSCLC tumorigenicity *in vivo*. Proc. Am. Assoc. Cancer Res., *43:* 816–817, 2002.

115. DeFatta, R. J., Nathan, C. A., and De Benedetti, A. Antisense RNA to eIF4E suppresses oncogenic properties of a head and neck squamous cell carcinoma cell line. Laryngoscope, *110:* 928–933, 2000.

116. Herbert, T. P., Fahraeus, R., Prescott, A., Lane, D. P., and Proud, C. G. Rapid induction of apoptosis mediated by peptides that bind initiation factor eIF4E. Curr. Biol., *10:* 793–796, 2000.

117. DeFatta, R. J., Li, Y., and De Benedetti, A. Selective killing of cancer cells based on translational control of a suicide gene. Cancer Gene Ther., *9:* 573–578, 2002.

TECHNICAL UPDATE
FROM YOUR LABORATORY SERVICES PROVIDER

HER-2/neu Breast Cancer Predictive Testing

Julie Sanford Hanna, Ph.D. and Dan Mornin, M.D.

EACH YEAR, OVER 182,000 WOMEN in the United States are diagnosed with breast cancer, and approximately 45,000 die of the disease.[1] Incidence appears to be increasing in the United States at a rate of roughly 2% per year. The reasons for the increase are unclear, but non-genetic risk factors appear to play a large role.[2]

Five-year survival rates range from approximately 65%-85%, depending on demographic group, with a significant percentage of women experiencing recurrence of their cancer within 10 years of diagnosis. One of the factors most predictive for recurrence once a diagnosis of breast cancer has been made is the number of axillary lymph nodes to which tumor has metastasized. Most node-positive women are given adjuvant therapy, which increases their survival. However, 20%-30% of patients without axillary node involvement also develop recurrent disease, and the difficulty lies in how to identify this high-risk subset of patients. These patients could benefit from increased surveillance, early intervention, and treatment.

Prognostic markers currently used in breast cancer recurrence prediction include tumor size, histological grade, steroid hormone receptor status, DNA ploidy, proliferative index, and cathepsin D status. Expression of growth factor receptors and over-expression of the HER-2/neu oncogene have also been identified as having value regarding treatment regimen and prognosis.

HER-2/neu (also known as c-erbB2) is an oncogene that encodes a transmembrane glycoprotein that is homologous to, but distinct from, the epidermal growth factor receptor. Numerous studies have indicated that high levels of expression of this protein are associated with rapid tumor growth, certain forms of therapy resistance, and shorter disease-free survival. The gene has been shown to be amplified and/or overexpressed in 10%-30% of invasive breast cancers and in 40%-60% of intraductal breast carcinoma.[3]

There are two distinct FDA-approved methods by which HER-2/neu status can be evaluated: immunohistochemistry (IHC, HercepTest™) and FISH (fluorescent in situ hybridization, PathVysion™ Kit). Both methods can be performed on archived and current specimens. The first method allows visual assessment of the amount of HER-2/neu protein present on the cell membrane. The latter method allows direct quantification of the level of gene amplification present in the tumor, enabling differentiation between low- versus high-amplification. At least one study has demonstrated a difference in recurrence risk in women younger than 40 years of age for low- versus high-amplified tumors (54.5% compared to 85.7%); this is compared to a recurrence rate of 16.7% for patients with no HER-2/neu gene amplification.[4] HER-2/neu status may be particularly important to establish in women with small (≤1 cm) tumor size.

The choice of methodology for determination of HER-2/neu status depends in part on the clinical setting. FDA approval for the Vysis FISH test was granted based on clinical trials involving 1549 node-positive patients. Patients received one of three different treatments consisting of different doses of cyclophosphamide, Adriamycin, and 5-fluorouracil (CAF). The study showed that patients with amplified HER-2/neu benefited from treatment with higher doses of adriamycin-based therapy, while those with normal HER-2/neu levels did not. The study therefore identified a sub-set of women, who because they did not benefit from more aggressive treatment, did not need to be exposed to the associated side effects. In addition, other evidence indicates that HER-2/neu amplification in node-negative patients can be used as an independent prognostic indicator for early recurrence, recurrent disease at any time and disease-related death.[5] Demonstration of HER-2/neu gene amplification by FISH has also been shown to be of value in predicting response to chemotherapy in stage-2 breast cancer patients.

Selection of patients for Herceptin© (Trastuzumab) monoclonal antibody therapy, however, is based upon demonstration of HER-2/neu protein overexpression using HercepTest™. Studies using Herceptin© in patients with metastatic breast cancer show an increase in time to disease progression, increased response rate to chemotherapeutic agents and a small increase in overall survival rate. The FISH assays have not yet been approved for this purpose, and studies looking at response to Herceptin© in patients with or without gene amplification status determined by FISH are in progress.

In general, FISH and IHC results correlate well. However, subsets of tumors are found which show discordant results; i.e., protein overexpression without gene amplification or lack of protein overexpression with gene amplification. The clinical significance of such results is unclear. Based on the above considerations, HER-2/neu testing at SHMC/PAML will utilize immunohistochemistry (HercepTest©) as a screen, followed by FISH in IHC-negative cases. Alternatively, either method may be ordered individually depending on the clinical setting or clinician preference.

CPT code information

HER-2/neu via IHC

88342 (including interpretive report)

HER-2/neu via FISH

88271×2 Molecular cytogenetics, DNA probe, each
88274 Molecular cytogenetics, interphase in situ hybridization, analyze 25-99 cells
88291 Cytogenetics and molecular cytogenetics, interpretation and report

Procedural Information

Immunohistochemistry is performed using the FDA-approved DAKO antibody kit, Herceptest©. The DAKO kit contains reagents required to complete a two-step immunohistochemical staining procedure for routinely processed, paraffin-embedded specimens. Following incubation with the primary rabbit antibody to human HER-2/neu protein, the kit employs a ready-to-use dextran-based visualization reagent. This reagent consists of both secondary goat anti-rabbit antibody molecules with horseradish peroxidase molecules linked to a common dextran polymer backbone, thus eliminating the need for sequential application of link antibody and peroxidase conjugated antibody. Enzymatic conversion of the subsequently added chromogen results in formation of visible reaction product at the antigen site. The specimen is then counterstained; a pathologist using light-microscopy interprets results.

FISH analysis at SHMC/PAML is performed using the FDA-approved PathVysion™ HER-2/neu DNA probe kit, produced by Vysis, Inc. Formalin fixed, paraffin-embedded breast tissue is processed using routine histological methods, and then slides are treated to allow hybridization of DNA probes to the nuclei present in the tissue section. The Pathvysion™ kit contains two direct-labeled DNA probes, one specific for the alphoid repetitive DNA (CEP 17, spectrum orange) present at the chromosome 17 centromere and the second for the HER-2/neu oncogene located at 17q11.2-12 (spectrum green). Enumeration of the probes allows a ratio of the number of copies of chromosome 17 to the number of copies of HER-2/neu to be obtained; this enables quantification of low versus high amplification levels, and allows an estimate of the percentage of cells with HER-2/neu gene amplification. The clinically relevant distinction is whether the gene amplification is due to increased gene copy number on the two chromosome 17 homologues normally present or an increase in the number of chromosome 17s in the cells. In the majority of cases, ratio equivalents less than 2.0 are indicative of a normal/negative result, ratios of 2.1 and over indicate that amplification is present and to what degree. Interpretation of this data will be performed and reported from the Vysis-certified Cytogenetics laboratory at SHMC.

References

1. Wingo, P.A., Tong, T., Bolden, S., "Cancer Statistics", 1995;45:1:8-31.
2. "Cancer Rates and Risks", 4th ed., National Institutes of Health, National Cancer Institute, 1996, p. 120.
3. Slamon, D.J., Clark, G.M., Song, S.G., Levin, W.J., Ullrich, A., McGuire, W.L. "Human breast Cancer: Correlation of relapse and survival with amplification of the her-2/neu oncogene". Science, 235:177-182, 1987.
4. Xing, W.R., Gilchrist, K.W., Harris, C.P., Samson, W., Meisner, L.F. "FISH detection of HER-s/neu oncogene amplification in early onset breast cancer". Breast Cancer Res. And Treatment 39(2):203-212, 1996.
5. Press, M.F. Bernstein, L., Thomas, P.A., Meisner, L.F., Zhou, J.Y., Ma, Y., Hung, G., Robinson, R.A., Harris, C., El-Naggar, A., Slamon, D.J., Phillips, R.N., Ross, J.S., Wolman, S.R., Flom, K.J., "Her-2/neu gene amplification characterized by fluorescence in situ hybridization: poor prognosis in node-negative breast carcinomas", J. Clinical Oncology 15(8):2894-2904, 1997.

Provided for the clients of

PATHOLOGY ASSOCIATES MEDICAL LABORATORIES
PACLAB NETWORK LABORATORIES
TRI-CITIES LABORATORY
TREASURE VALLEY LABORATORY

For more information, please contact your local representative.

Evaluation of HER-2/*neu* Gene Amplification and Overexpression: Comparison of Frequently Used Assay Methods in a Molecularly Characterized Cohort of Breast Cancer Specimens

By Michael F. Press, Dennis J. Slamon, Kerry J. Flom, Jinha Park, Jian-Yuan Zhou, and Leslie Bernstein

Purpose: To compare and evaluate HER-2/*neu* clinical assay methods.

Materials and Methods: One hundred seventeen breast cancer specimens with known HER-2/*neu* amplification and overexpression status were assayed with four different immunohistochemical assays and two different fluorescence in situ hybridization (FISH) assays.

Results: The accuracy of the FISH assays for HER-2/*neu* gene amplification was high, 97.4% for the Vysis PathVision assay (Vysis, Inc, Downers Grove, IL) and 95.7% for the the Ventana INFORM assay (Ventana, Medical Systems, Inc, Tucson, AZ). The immunohistochemical assay with the highest accuracy for HER-2/*neu* overexpression was obtained with R60 polyclonal antibody (96.6%), followed by immunohistochemical assays performed with 10H8 monoclonal antibody (95.7%), the Ventana CB11 monoclonal antibody (89.7%), and the DAKO HercepTest (88.9%; Dako, Corp, Carpinteria, CA). Only the sensitivities, and therefore, overall accuracy, of the DAKO Herceptest and Ventana CB11 immunohistochemical assays were significantly different from the more sensitive FISH assay.

Conclusion: Based on these findings, the FISH assays were highly accurate, with immunohistochemical assays performed with R60 and 10H8 nearly as accurate. The DAKO HercepTest and the Ventana CB11 immunohistochemical assay were statistically significantly different from the Vysis FISH assay in evaluating these previously molecularly characterized breast cancer specimens.

J Clin Oncol 20:3095-3105.

A LARGE NUMBER of molecular genetic alterations have now been identified in human breast cancers. These include both inherited and acquired genetic alterations. Among acquired alterations HER-2/*neu* gene amplification has received a great deal of attention recently. This is because of its potential clinical utility as a prognostic marker and as a predictor of responsiveness to treatment.

HER-2/*neu* (c-*erb*B-2) gene amplification, identified in 20% to 30% of breast cancers, is a prognostic marker of poor clinical outcome in node-negative and node-positive breast cancer and a predictor of lack of responsiveness to tamoxifen antiestrogen therapy and responsiveness to adjuvant doxorubicin chemotherapy. The development of an immunotherapeutic, trastuzamab (Herceptin; Genentech, Inc, South San Francisco, CA), to the extracellular domain of HER-2/*neu* has provided a new treatment for women with metastatic, HER-2/*neu*-overexpressing breast cancer. The importance of HER-2/*neu* in breast cancer treatment decision making has focused attention on the ability of various clinical assays to correctly assign HER-2/*neu* amplification and overexpression status.

One of us (D.J.S.) first identified HER-2/*neu* gene amplification in breast cancers 15 years ago using Southern hybridization of frozen breast cancer specimens.[1] Subsequently, we have shown a correlation between gene amplification and an increased level of HER-2/*neu* expression, referred to as overexpression, at the mRNA and protein product levels.[2] Overexpression of HER-2/*neu* in cultured cells and transgenic mice is associated with malignant transformation in the cells overexpressing this gene.[3-5] Although initially controversial, we and others have shown that HER-2/*neu* gene amplification or overexpression predicts shorter disease-free survival or shorter overall survival in both axillary lymph node–negative[6-17] and node-positive breast cancer.[1,2,8,18,19] In addition, HER-2/*neu* overexpression has been associated with responsiveness to cytotoxic chemotherapy[12,20-25] and resistance to tamoxifen antiestro-

From the Breast Cancer Research Program of the Lee Breast Center, Department of Pathology and Department of Preventive Medicine, Norris Comprehensive Cancer Center, University of Southern California; Division of Hematology and Oncology, University of California Los Angeles School of Medicine, Los Angeles, CA; and Vysis, Inc, Downers Grove, IL.

Submitted September 21, 2001; accepted April 23, 2002.

Supported by grant no. CA48780 from the National Cancer Institute, grant no. NO1-HD-3-3175 from the National Institute for Child Health and Human Development, Bethesda, MD, and grant no. BCRP 5EB-3101 from the California Breast Cancer Research Program, Oakland, CA. J.P. was supported by grant no. DAMD17-97-1-7161 from the United States Army Medical Research and Materiel Command, Fort Detrick, MD.

Address reprint requests to: Michael F. Press, MD, PhD, Norris Topping Tower Room 5409, 1441 Eastlake Ave, U.S.C./Norris Comprehensive Cancer Center, Los Angeles, CA 90033; email: press@hsc.usc.edu.

0732-183X/02/2014-3095/$20.00

gen therapy.[26-29] Experimental work with animal models and clinical trials with women having HER-2/*neu*-overexpressing breast cancer have shown tumor regression in response to anti–HER-2/*neu* antibody therapy. Recently, the United States Food and Drug Administration (FDA) approved an immunotherapeutic agent for women with metastatic, HER-2/*neu*-overexpressing breast cancers. This immunotherapeutic is a recombinant, humanized anti–HER-2/*neu* monoclonal antibody, trastuzamab. The FDA has also approved two fluorescence in situ hybridization (FISH) assays for clinical testing of HER-2/*neu* gene amplification (Ventana Medical Systems, Inc, Tucson, Az, and Vysis, Inc, Downers Grove, IL) and two immunohistochemical assays for HER-2/*neu* overexpression (DAKO, Corp, Carpinteria, CA, and Ventana Medical Systems, Inc). The FDA approval for gene amplification tests was originally for identification of women with node-negative disease at high-risk for recurrence or disease-related death or for selection to doxorubicin chemotherapy. A recently approved application to the FDA broadened the indications for FISH testing to include selection of women with metastatic breast cancer for trastuzamab therapy. Both immunohistochemistry assays are approved for selection of women with metastatic breast cancer to receive trastuzamab treatment.

Previous molecular analyses of frozen breast cancer specimens indicate that amplification is closely associated with overexpression;[2,18] however, the detection rates for immunohistochemistry reported by clinical laboratories are different from one another and, for at least one of the immunohistochemical assays, higher than expected.[30,31] This apparent discrepancy has generated considerable confusion among clinicians, pathologists, and patients regarding appropriate methods for HER-2/*neu* testing. To address this issue, we have used each of the four FDA-approved assays and two additional immunohistochemical assays to test for HER-2/*neu* alterations in breast cancer specimens in which we previously determined amplification and overexpression levels using solid matrix blotting techniques.

MATERIALS AND METHODS

Breast Cancer Specimens

Breast cancer specimens were selected for inclusion in this study based on the following criteria: (1) specimens were previously molecularly characterized for HER-2/*neu* gene amplification and overexpression using solid matrix blotting techniques,[2,6,18,32] and (2) the analyses of amplification and expression showed agreement between amplification status and expression status. To minimize inclusion of tumors that were misclassified because of dilutional artifacts associated with solid matrix blotting methods, samples selected as representative of gene amplification were required to show both amplification and overexpression, whereas cases selected as representative of nonamplified samples were required to show neither gene amplification nor overexpression by solid matrix blotting techniques. Tumors showing a disagreement between the amplification and expression status were excluded from the analysis.

HER-2/*neu* gene amplification was previously determined by solid matrix blotting (either Southern hybridization or slot/dot blot hybridization) using DNA extracted from breast cancer specimens.[2,11] HER-2/*neu* expression was assessed by either solid matrix blotting of RNA or protein extracted from breast cancer specimens (Northern hybridization, dot blot hybridization, and Western immunoblot) or immunohistochemistry. If expression was assessed by more than one method, then the expression level was assigned according to the classification determined by the majority of assay methods. Ninety-eight cases had expression data available from two or more assay methods.[2,18] The remaining 19 cases had expression data available from only a single assay method.[6]

Breast cancer specimens with known HER-2/*neu* gene copy and expression levels were evaluated in this study as archival, paraffin-embedded tissue specimens. The specimens were arrayed in two paraffin-embedded, multitumor specimen blocks, as described elsewhere in detail.[32] The histopathology of all specimens remaining in the multispecimen blocks was reviewed, and only specimens containing breast carcinoma cells were included in the study. One hundred seventeen cases met all of the above criteria and were included in this study with 43 breast cancers showing both amplification and overexpression and 74 breast cancers showing neither amplification nor overexpression. The multitumor specimen block approach permitted the use of small quantities of reagents and, more importantly, assured equal exposure of each breast cancer to all of the reagents in the assay system.

*FISH for Gene Amplification Using Both HER-2/*neu *and Chromosome 17 Centromere Probes*

HER-2/*neu* gene copy level was determined by FISH in paraffin-embedded tissue sections as a ratio of HER-2/*neu* gene copies-to-chromosome 17 centromere copies (Vysis, Inc). This approach eliminates increased gene copy number due to aneuploidy or aneusomy alone. Because the HER-2/*neu* gene is located on chromosome 17,[33] an alpha satellite (pericentromeric) DNA probe for chromosome 17 was selected as an internal control for chromosomal aneuploidy. By comparing the number of copies of these two chromosomal markers, aneuploidy of chromosome 17 is excluded as a source of increased HER-2/*neu* gene copy number. The alpha-satellite DNA is also used as an internal control to correct for differences that might arise as a result of tissue sectioning artifacts in paraffin-embedded section preparation.

FISH was performed according to the PathVysion (Vysis, Inc) protocol, which is described in the package insert as approved by the United States FDA. In brief, the PathVysion protocol involved rehydration of paraffin-embedded, 4-μm thick, multitumor tissue sections through Hemo-De clearing agent (Vysis, Inc) and a graded series of alcohols. The sections were air dried, pretreated (80°C, 30 minutes), and digested with protease (37°C, 10 to 20 minutes) before hybridizing with fluorescent-labeled probes for HER-2/*neu* gene and alpha-satellite DNA for chromosome 17. The locus specific identifier (LSI) HER-2/*neu* DNA probe is a 190-Kb SpectrumOrange (Abbott-Vysis, Inc) directly labeled fluorescent DNA probe specific for the HER-2/*neu* gene locus (17q11.2-q12). The chromosome enumeration probe (CEP) 17 DNA probe is a 5.4-Kb SpectrumGreen (Abbott-Vysis, Inc) directly labeled fluorescent DNA probe specific for the alpha satellite DNA sequence at the centromeric region of chromosome 17 (17p11.1-q11.1).

The probes were premixed and predenatured in hybridization buffer for ease of use.

The nuclei were routinely counterstained with an intercalating fluorescent counterstain, 4'-6'-diamidino-2'-phenylindole (DAPI). Staining was visualized with a Zeiss Axioskop 20 fluorescence microscope (Carl Zeiss, Inc, Oberkochen, Germany). SK-BR-3 human breast cancer cells, known to have HER-2/*neu* gene amplification, and MDA-MB-468 human breast cancer cells, known to lack HER-2/*neu* gene amplification, were used as control cells (American Type Culture Collection, HTB30 and HTB132)[34] (unpublished data). For each tumor, 60 tumor cell nuclei were identified and scored for both HER-2/*neu* and chromosome 17 centromere number. Using criteria established for Southern hybridization,[2,16,18] HER-2/*neu* gene amplification was defined as a HER-2/*neu*-to-chromosome 17 ratio greater than 2.0 as required by the manufacturer.

*FISH to Evaluate Gene Amplification Using Only a HER-2/*neu *Probe*

An indirect method for localization of the HER-2/*neu* gene by FISH, Ventana InformHer (Ventana Medical Systems, Inc), was performed as previously described[7] and as approved by the FDA. Briefly, sections of formalin-fixed, paraffin-embedded breast cancer tissue were mounted on microscope slides and pretreated chemically (Pretreatment step, reduction of peptide disulfide bonds) and enzymatically (Protein digestion step, digestion of proteins) to remove proteins that block DNA access. The DNA in the sections was converted from double to single strands by denaturation at 75°C using a mixture of 20× saline sodium citrate and formamide. A hybridization solution, containing a digoxigenin-labeled DNA probe complementary to the HER-2/*neu* gene sequence, was applied to the tissue sections. Incubation was carried out under conditions favorable for annealing of probe DNA and genomic DNA sequences as described by the manufacturer (Ventana, Inc). Unannealed probe was washed off using a mixture of 20× saline sodium citrate and formamide. The hybridized probe was detected using a fluorescein-labeled avidin, which bound to biotin on the DNA probe, thereby immobilizing the fluorescein at the site of the HER-2/*neu* gene. The remainder of the DNA was then stained with DAPI in Antifade. Excitement of fluorescein and DAPI by light from a mercury arc lamp with appropriate filters in a Zeiss fluorescence microscope resulted in the emission of green and blue light, respectively. The observer selected these two colors by using a microscope filter set designed for simultaneous viewing of DAPI and fluorescein, and scored nuclei in the tissue section for the number of green signals in blue nuclei.

For each specimen, gene copy level was assessed in two areas of 20 nonoverlapping tumor cell nuclei. A tumor was amplified if there were more than four copies of HER-2/*neu* per cell, as described elsewhere[7] and as required by the manufacturer (Ventana, Inc). Staining was visualized with a Zeiss fluorescence microscope. SK-BR-3 human breast cancer cells, known to have HER-2/*neu* gene amplification, and MDA-MB-468 human breast cancer cells, known to lack HER-2/*neu* gene amplification, were used as control cells.

*Immunohistochemical Assay for HER-2/*neu *Protein Using the R60 Polyclonal Antibody*

Rabbit polyclonal antibody (R60) was used with the peroxidase antiperoxidase immunohistochemical technique to demonstrate HER-2/*neu* protein product in tissue sections, as described in detail elsewhere.[2,6,32] We have demonstrated the specificity of the primary R60 antibody for HER-2/*neu* receptor protein with western immunoblot analyses and competition studies performed using the peroxidase antiperoxidase technique.[18] This immunohistochemical technique involved incubation of the tissue sections with the following three different antibodies: a primary rabbit R60 HER-2/*neu* antibody (1:1000 dilution, overnight incubation at 2°C); a secondary or bridging goat anti-immunoglobulin G antibody (1:50 dilution, 30 minutes, room temperature)(Zymed, Inc, South San Francisco, CA); and, finally, a rabbit peroxidase antiperoxidase antibody with bound horseradish peroxidase (1:50 dilution, 30 minutes, room temperature)(Stemberger, Inc, Lutherville, MD). The site of immunoprecipitates was identified using the chromogen, diaminobenzidine, which can be visualized microscopically after reaction with horseradish peroxidase. The tissue sections were counterstained with ethyl green and evaluated with an Olympus BH-2 bright field microscope (Olympus America, Inc, Melville, NY) as showing low expression or overexpression according to subjective criteria described elsewhere.[6,32]

*Immunohistochemical Assay for HER-2/*neu *Protein Using the 10H8 Monoclonal Antibody*

Mouse monoclonal antibody to the protein product of the proto-oncogene HER-2/*neu* (c-*erb*B2) was used with the peroxidase antiperoxidase immunohistochemical technique to demonstrate this protein product in tissue sections. The immunohistochemical technique involved incubation of the tissue sections with two different antibody reagents: the primary mouse antibody specific for HER-2/*neu* receptor protein (10H8 monoclonal antibody, 5 μg/mL, 30 minutes, room temperature), followed by a goat antimouse immunoglobulin conjugated to a HRP-labelled dextran polymer (Envision+, 30 minutes, room temperature; DAKO, Corp). The site of immunoprecipitates was identified using a chromogen, diaminobenzidine, visualized microscopically. Prediluted goat antimouse immunoglobulin G conjugated to HRP-labelled dextran polymer (Envision+) was used as specified by the manufacturer (DAKO Corp, catalog no. K4001). The specificity of the primary antibody for HER-2/*neu* receptor protein has been previously demonstrated with western immunoblot analysis, immunoprecipitation assays and competition studies performed using the peroxidase antiperoxidase technique.[35] Immunostaining was scored subjectly with an Olympus bright-field microscope as strong continuous membrane staining (high immunostaining), moderate continuous membrane staining (medium immunostaining), weak barely detectable membrane staining (low immunostaining), and an absence of membrane staining (low immunostaining). Strong and moderate membrane staining were considered to represent overexpression, and no membrane staining or weak membrane staining was considered to represent low HER-2/*neu* expression.

*DAKO HercepTest for Immunoenzymatic Staining to Detect HER-2/*neu *Protein*

The HercepTest (DAKO, Corp) is a subjectively scored immunohistochemical assay used to determine HER-2/*neu* protein overexpression in histologic sections of breast cancer specimens. The HercepTest is approved by the FDA (September, 1998) for selection of women with breast cancer to receive trastuzumab humanized monoclonal antibody therapy. In this study, the HercepTest was performed according to the approved protocol as described by the manufacturer. Tissue sections were cut, mounted on plus slides, heat-treated for antigen retrieval, and immunostained. Antigen retrieval involved boiling the tissue sections at 95°C to 99°C in 10 mmol/L citrate buffer for 40 minutes. The

sections were cooled and treated with peroxidase-blocking reagent for 5 minutes, rinsed, and treated with sufficient primary rabbit HER-2/*neu* antibody to cover the entire tissue section for 30 minutes. The sections were rinsed again and treated for 30 minutes with visualization reagent, a solution containing both secondary goat antirabbit antibody and horseradish peroxidase linked to a common dextran polymer backbone. After rinsing away excess visualization reagent, the sections were incubated in diaminobenzidine for 10 minutes to identify the location of immunoprecipitates. The tissue sections were processed with the DAKO Autostainer Universal Staining System according to the instructions of the manufacturer (DAKO, Corp). The sections were counterstained with hematoxylin and mounted in Permount. Immunostaining was interpreted with a bright-field Olympus microscope according to the scoring system of the manufacturer as 0, 1+, 2+, and 3+ (DAKO, Corp); 2+ and 3+ immunostaining was considered to be overexpression and 0/1+ immunostaining was considered to be low expression.

*Immunohistochemical Assay for HER-2/*neu *Protein Using the CB11 Monoclonal Antibody*

The protocol and instructions provided by Ventana Medical Systems, Inc, were used for the CB11 immunohistochemical assay under the supervision of a Ventana employee. Prediluted anti–HER-2/*neu* monoclonal antibody CB11 (Ventana Medical Systems, Inc) was used with ready-to-use detection kits and a Ventana Automated Slide Stainer according to the manufacturer's specifications to detect HER-2/*neu*. Each antigen-retrieved slide was labeled with the appropriate bar code specifying the staining procedure. The sequence of staining procedures carried out by the automated slide stainer included application of inhibitor solution to decrease endogenous peroxidase activity (4 minutes, 37°C), application of primary antibody (CB11, prepared by the manufacturer at approximately 0.63 μg/mL, 32 minutes, 37°C), application of biotinylated secondary antibody (8 minutes, 37°C), application of avidin/streptavidin-enzyme conjugate (8 minutes, 37°C), and application of diaminobenzidine chromogenic substrate. The specimen processing was supervised by a representative of the manufacturer to ensure that the tissue was processed appropriately. The immunostaining was scored 0, 1+, 2+, and 3+, with 2+ and 3+ considered overexpression, as described previously.[36-38]

Statistical Analyses

For each of the six assay methods, the sensitivity, specificity, and accuracy were calculated using the known HER-2/*neu* status of the tumor as the gold standard. Exact binomial 95% confidence intervals (CI) were calculated for each of these measures. To measure the degree of concordance (or accuracy corrected for agreement by chance), the kappa statistic and its 95% CI were calculated.[39] In general, values of kappa greater than or equal to 0.91 are considered to represent almost perfect agreement with 1.0 being perfect agreement.[40,41] The sensitivity and accuracy measures were compared using Fisher's exact test.

RESULTS

One hundred seventeen breast cancer specimens with known HER-2/*neu* amplification and overexpression status determined by solid matrix blotting techniques and immunohistochemistry in previous studies[2,6,11] were reanalyzed as paraffin-embedded tissue sections for gene amplification using two FISH methods and for HER-2/*neu* overexpression using four different immunohistochemical assays.

*FISH for Gene Amplification Using Both HER-2/*neu *and Chromosome 17 Centromere Probes*

The average HER-2/*neu* gene copy number and chromosome 17 centromere copy number were determined in 60 tumor cell nuclei from each of the 117 breast cancer specimens (Fig 1). The HER-2/*neu*-to-chromosome 17 ratios varied from 0.69 to 19.07; values greater than or equal to 2.0 demonstrate gene amplification. Forty-two breast cancers were amplified in this assay, and 75 were not. After the results were unmasked, the assay sensitivity was 95.4% (41 of 43 breast cancers), and the specificity was 98.6% (73 of 74) (Table 1). Two breast cancers, known to have gene amplification, had HER-2/*neu*-to-chromosome 17 centromere ratios of 1.98 and 1.89. Another breast cancer, known to lack gene amplification, had a HER-2/*neu*-to-chromosome 17 centromere ratio of 4.06. One hundred fourteen of the 117 breast carcinomas were correctly categorized, for an accuracy rate of 97.4%. The degree of concordance (kappa) was 0.945 (95% CI, 0.883 to 1.0).

*FISH to Evaluate Gene Amplification Using Only a HER-2/*neu *Probe*

The number of HER-2/*neu* gene copies was determined in 20 tumor cell nuclei for each of two microscopic fields in the 117 breast cancer specimens using FISH with a digoxigenin-labeled cosmid probe (Ventana, Inc) (Fig 1). The average number of HER-2/*neu* gene copies per tumor cell nucleus ranged from 1.28 to 25.50; values greater than or equal to 4.0 demonstrate gene amplification. The sensitivity of this FISH assay was 95.4% (41 of 43 breast cancers); the specificity was 96.0% (71 of 74); and the accuracy was 95.7% (112 of 117) (Table 1). The degree of concordance (kappa) was 0.909 (95% CI, 0.830 to 0.987). Forty-four of the 117 breast cancers had more than 4.0 HER-2/*neu* gene copies per tumor cell nucleus, and 73 had less than 4.0 HER-2/*neu* gene copies per tumor cell nucleus. One of the three cases with a false-positive result was the same case as the single false-positive result found with the Vysis FISH assay (average of 8.30 HER-2/*neu* gene copies per tumor cell nucleus). The other two false-positive results were above the cutoff of 4.0 (4.93 and 4.38). The two false-negative cases had averages of 3.58 and 3.97 HER-2/*neu* gene copies per tumor cell nucleus. These were the same two cases identified as false-negatives with the Vysis FISH assay (above).

*Immunohistochemical Assay for HER-2/*neu *Protein Using the R60 Polyclonal Antibody*

Overexpression of HER-2/*neu* protein was identified in 39 of the breast carcinomas. These breast cancers had membrane staining above the minimal low amount of

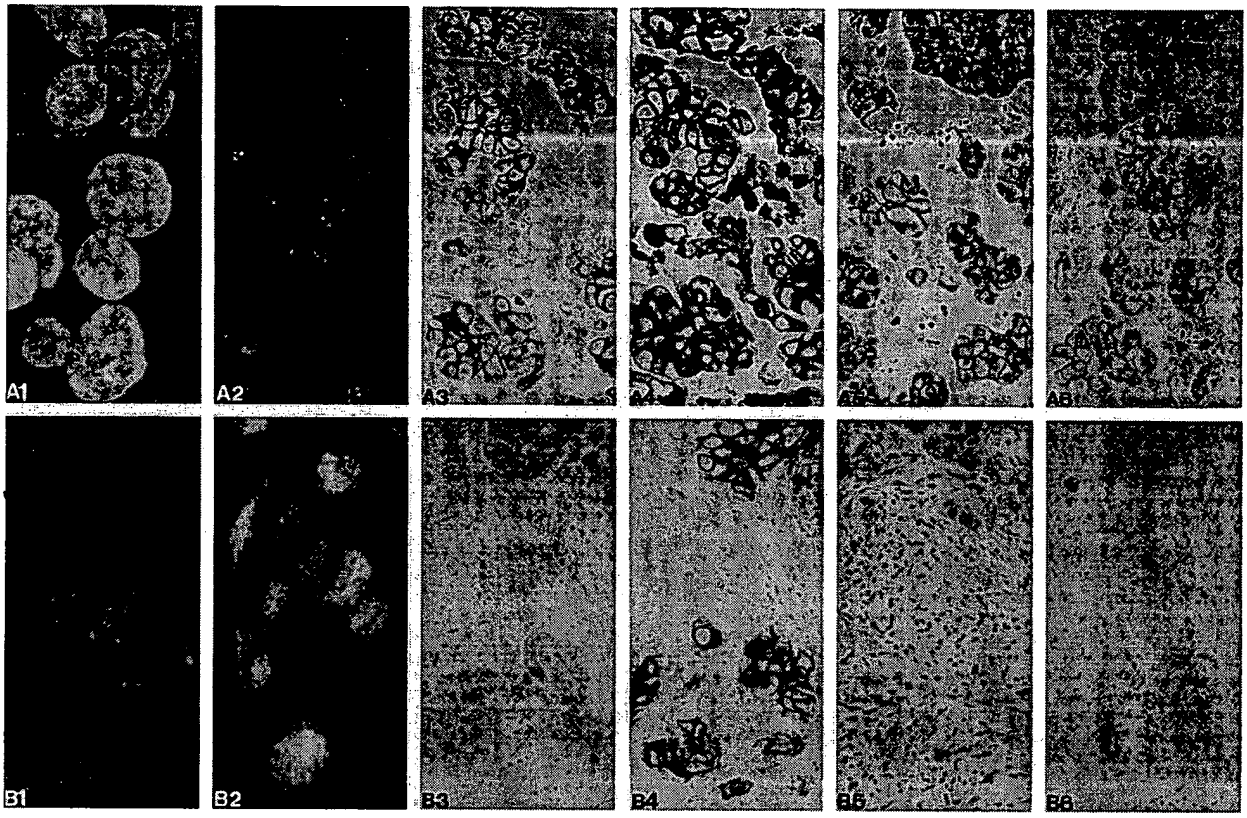


Fig 1. Comparison of FISH and immunohistochemical staining. The FISH and immunostaining results are illustrated for two breast cancers (A and B) known to have gene amplification and overexpression. In one breast cancer (A) (MTB#1, 3-3), all assay methods (A1-A6) demonstrated positive HER-2/*neu* results. In the other breast cancer (B) (MTB#1, 2-6), both FISH assays (B1, B2) and one immunohistochemical assay (B4) demonstrated positive HER-2/*neu* results. (A1 and B1) Vysis PathVysion FISH assay. (A2 and B2) Ventan InformHer FISH assay. (A3 and B3) Rabbit R60 polyclonal antibody immunohistochemical assay. (A4 and B4) Mouse 10H8 monoclonal antibody immunohistochemical assay. (A5 and B5) DAKO HercepTest immunohistochemical assay. (A6 and B6) Ventana CB11 immunohistochemical assay.

membrane staining observed in normal epithelial cells, and these cases were scored as having medium- or high-intensity membrane staining, as described in Materials and Methods and elsewhere (Fig 1).[2,6,32] Seventy-eight of the breast carcinomas were scored as having low expression because either no membrane staining or weak membrane immunostaining was observed. The sensitivity of immunohistochemistry with R60 polyclonal antibody was 90.6% (39 of 43 breast cancers); the specificity was 100% (74 of 74); and the accuracy was 96.6% (113 of 117) (Table 1). The degree of concordance (kappa) was 0.925 (95% CI, 0.853 to 0.997).

Table 1. Comparison of FISH and Immunohistochemical Assay Results Using 117 Breast Cancers With Known HER-2/neu Gene Amplification and Overexpression Status

Assay	No. Positive/ No. Negative	Sensitivity	95% CI	Specificity	95% CI	Kappa	95% CI	Accuracy	95% CI
FISH, Vysis	42/75	41/43 = 0.954	0.842-0.994	73/74 = 0.986	0.927-1.0	0.945	0.883-1.0	0.974	0.927-0.995
FISH, Ventana	44/73	41/43 = 0.954	0.842-0.994	71/74 = 0.960	0.886-0.992	0.909	0.830-0.987	0.957	0.903-0.986
IHC-R60	39/78	39/43 = 0.907	0.779-0.974	74/74 = 1.00	0.951-1.0	0.925	0.853-0.997	0.966	0.915-0.991
IHC-10H8	38/79	38/43 = 0.884	0.749-0.961	74/74 = 1.00	0.951-1.0	0.906	0.825-0.986	0.957	0.903-0.986
IHC-CB11	31/86	31/43 = 0.721	0.563-0.847*	74/74 = 1.00	0.951-1.0	0.766	0.644-0.888	0.897	0.828-0.946*
IHC-DAKO	30/87	30/43 = 0.698	0.539-0.828*	74/74 = 1.00	0.951-1.0	0.745	0.618-0.871	0.889	0.817-0.939*

Abbreviation: IHC, immunohistochemistry (manual).

*Results differ significantly from those obtained with Vysis FISH assay ($P < .05$).

*Immunohistochemical Assay for HER-2/*neu *Protein Using the 10H8 Monoclonal Antibody*

Membrane immunostaining was scored as described in Materials and Methods using low-, medium-, and high-intensity categories, with medium and high immunostaining corresponding to overexpression (Fig 1). Thirty-eight of the 117 breast cancers were considered to have medium- or high-intensity immunostaining, whereas 79 were considered to have low immunostaining. When the known HER-2/*neu* expression status was unmasked, 38 of the 43 breast cancers with overexpression and all 74 of the low-expression breast cancers had been correctly categorized. The sensitivity of immunohistochemistry with 10H8 monoclonal antibody was 88.4% (38 of 43 breast cancers); the specificity was 100% (74 of 74); and the accuracy was 95.7% (112 of 117) (Table 1). The degree of concordance (kappa) was 0.906 (95% CI, 0.825 to 0.986).

*DAKO HercepTest for Immunoenzymatic Staining to Detect HER-2/*neu *Protein*

Immunostaining of HER-2/*neu* oncoprotein by the HercepTest kit was assessed as 0, 1+, 2+, and 3+, as described by the manufacturer, with 0 and 1+ scored as low expression and 2+ and 3+ scored as overexpression. Seventeen breast cancers had 3+ immunostaining, 13 had 2+ immunostaining, five had 1+ immunostaining, and 82 had no immunostaining. Unmasking of the known expression levels demonstrated that 30 of 43 breast cancers with overexpression and all 74 breast cancers with low expression had been correctly categorized. The sensitivity was 69.8% (30 of 43 breast cancers); the specificity was 100% (74 of 74); and the accuracy was 88.9% (104 of 117). The degree of concordance (kappa) was 0.745 (95% CI, 0.618 to 0.871). The HercepTest would have had a substantially higher false-negative rate if only the 3+ breast cancers had been considered positive (sensitivity, 13 of 43 = 30.2%; false-negatives = 69.8%).

*Immunohistochemical Assay for HER-2/*neu *Protein Using the CB11 Monoclonal Antibody*

Immunostaining with the CB11 monoclonal antibody was evaluated as recommended by the manufacturer (Ventana, Inc). Thirty-one breast cancers were assessed as having HER-2/*neu* overexpression, and 86 were assessed as having low expression. Unmasking of the known expression levels demonstrated that 31 of the 43 known overexpressors and all 74 of the known low expressors had been correctly categorized. Five breast cancers had 1+ immunostaining; four of these breast cancers were known amplified overexpressors. The sensitivity of immunohistochemistry with the CB11 monoclonal antibody was, therefore, 72.1% (31 of 43 breast cancers); the specificity was 100% (74 of 74); and the accuracy was 89.7% (105 of 117). The degree of concordance (kappa) was 0.766 (95% CI, 0.644 to 0.888). If any degree of membrane staining (1+, 2+, or 3+) was considered overexpression, then the specificity was 81.4% (35 of 43); the sensitivity was 98.6% (73 of 74), and the accuracy was 92.3% (108 of 117).

Comparison of FISH and Immunohistochemical Assays

Overall, the Vysis FISH assay was the most accurate, followed by the R60 immunohistochemical assay, the Ventana FISH assay, the 10H8 immunohistochemical assay, the Ventana CB11 immunohistochemical assay, and the DAKO HercepTest (Table 1). In our hands, the immunohistochemical assays had perfect specificity but they had lower sensitivity. Because of 12 Ventana (considering 2+ and 3+ as overexpression) or 13 DAKO Herceptest immunohistochemistry false-negative results, the results for the immunohistochemical DAKO HercepTest and immunohistochemical Ventana CB11 assay were significantly lower than the Vysis FISH assay with regard to sensitivity and, therefore, accuracy.

The same two cases, known to be amplified by previous analyses, were not scored as amplified with either the Vysis FISH assay (ratios less than 2.0; ie, 1.98 and 1.89 by the Vysis PathVision assay system) or the Ventana FISH assay (HER-2/*neu* gene copy levels less than 4.0; ie, 3.58 and 3.97). Both of these cases showed moderate (2+) immunostaining with the polyclonal R60 immunohistochemical assay but not with any of the other immunohistochemical assays. One breast cancer, known to lack amplification by previous analyses, was evaluated as amplified with both FISH assays (HER-2/*neu*-to-chromosome 17 ratio of 4.06 by the Vysis assay and a HER-2/*neu* gene copy number of 8.30 by the Ventana assay). Membrane immunostaining was detected in this case by 10H8 monoclonal antibody and the HercepTest (1+) but was not sufficiently strong to be scored as overexpression by either assay. A second breast cancer, known to lack amplification by previous analyses, was evaluated as amplified with only one of the two FISH assays (a HER-2/*neu* gene copy number of 4.93 by the Ventana assay). None of the immunohistochemical assays demonstrated any membrane immunostaining in this breast cancer.

Detection at Various Amplification/Overexpression Levels

Overall, those breast cancers with the highest level of gene amplification and overexpression (greater than 20-fold amplification of HER-2/*neu*) were identified the most frequently by each of the assay methods with one exception

Table 2. Detection With Each Assay Method by Known HER-2/neu Gene Amplification/Overexpression Status

Assay Method	> 20-Fold Amplification (n = 11)		5- to 20-Fold Amplification (n = 16)		2- to 5-Fold Amplification (n = 16)	
	No.	%	No.	%	No.	%
FISH (Vysis)	11	100	16	100	14	87.5
FISH (Ventana)	11	100	16	100	14	87.5
IHC-R60	10	90.9	14	87.5	15	93.8
IHC-10H8	11	100	15	93.8	12	75.0
IHC-DAKO	10	90.9	13	81.3	7	43.8
IHC-CB11	9	81.8	13	81.3	9	56.3

(Table 2). Those breast cancers with lower levels of gene amplification (two- to five-fold amplification) had a significantly lower level of detection, especially with two of the immunohistochemical assay methods evaluated manually as 2+ or 3+ membrane immunostaining (Table 2).

DISCUSSION

The potential role of HER-2/*neu* gene amplification and/or overexpression in the clinical management of women with breast cancer has been controversial since the first report.[1] The initial debate was whether HER-2/*neu* alterations correlated with poor clinical outcome in any group of women with breast cancer.[42-45] Subsequently, HER-2/*neu* overexpression was accepted as a predictor of disease-free survival or overall survival in women with node-positive breast cancer.[46] Although some studies clearly demonstrate that node-negative patients with HER-2/*neu* amplification/overexpression also have shorter disease-free survival or overall survival,[6-9,16] others have disputed this association.[23,45,47,48] Some investigators have suggested that the differences in clinical outcome observed among patients with HER-2/*neu*-overexpressing node-positive breast cancer are associated not with biologic behavior of the breast cancer cells but rather with responsiveness to treatment,[46] accounting for the differences observed in node-negative and node-positive breast cancers. However, several studies of clinical outcome among women with node-negative breast cancer treated solely by surgery demonstrated significant survival benefits associated with low expression of HER-2/*neu* compared with HER-2/*neu* overexpression.[6,7,14,16] Lack of agreement among these studies may also be related to the differing sensitivities and specificities of the assay methods used to assess HER-2/*neu* as a prognostic marker[6,32] and to lack of statistical power of some of the studies.[6,8] Although HER-2/*neu* gene amplification or overexpression seems to be a marker of poor prognosis in both node-negative and node-positive breast cancer, the major interest in this alteration is related to its utility as a predictive marker of responsiveness to treatment.

Some studies have shown HER-2/*neu* overexpression predicts responsiveness to high-dose doxorubicin chemotherapy,[20,25] lack of responsiveness to cyclophosphamide, methotrexate, and fluorouracil chemotherapy,[12,23] and lack of responsiveness to tamoxifen therapy.[26,28] However, HER-2/*neu*'s role as a predictor of responsiveness to therapy has been questioned because these initial observations have not been confirmed in some subsequent investigations.[24,49] Recently, a recombinant humanized, anti–HER-2/*neu* antibody, known as trastuzamab, was approved by the FDA for treatment of women with metastatic breast cancer whose tumors overexpress HER-2/*neu*. Although trastuzamab has shown efficacy in this clinical setting, the response rate has been variable in the initial clinical trials.[50-52]

A major problem when interpreting the published studies of HER-2/*neu* in the clinical setting is that different methods have been used to evaluate HER-2/*neu* alterations. These methodologic differences likely contribute substantially to these conflicting results and, therefore, conflicting conclusions related to HER-2/*neu*. The primary method used in the majority of these published studies is immunohistochemistry using archival, paraffin-embedded tissue blocks. However, this method is not performed consistently across laboratories. Furthermore, few of these laboratories have validated their immunohistochemical staining assessments using standardized specimens with known molecular changes. We previously demonstrated that available antibodies commonly used for immunohistochemistry have a wide range of sensitivities when used in molecularly characterized, archival, paraffin-embedded specimens.[32] None of the tested antibodies identified all of the known HER-2/*neu*–amplified, overexpression breast cancers. These antibodies were developed from animals immunized with native, nonfixed HER-2/*neu* protein as antigen. Therefore, it is not surprising that HER-2/*neu* would not be recognized in some formalin-fixed breast cancer specimens because none of the HER-2/*neu* antibodies were produced using formalin-fixed HER-2/*neu* protein as antigen.

Our previous evaluation of HER-2/*neu* antibodies[32] has been criticized because epitope retrieval methods were not used in our evaluation, the peroxidase antiperoxidase technique was used with all antibodies, and small samples of each tissue specimen were used.[31,53,54] The primary goal of our previous study was to demonstrate that differences in antibody sensitivity could account for the differing conclusions about HER-2/*neu*'s clinical utility described among published articles. At the time our study was conducted, none of the published articles had used heat-induced epitope retrieval as part of the immunohistochemical procedure. As we described in our article, "although the sensitivities of various antibodies have been characterized under the described routine conditions permitting more rational selection of immunostaining reagents, it is anticipated that there will be further improvements in HER-2/*neu* sensitivity in paraffin-embedded tissues as more antibodies become available for testing and as techniques for immunostaining improve. For example, the sensitivity of several of these antibodies is enhanced by 'antigen retrieval' with microwave pretreatment of the tissue sections".[32] We consider the use of a single immunostaining detection system in the previous study to be important for comparison between different antibodies on an equal basis. Therefore, the identified differences are due to differences in the primary antibodies and not due to differences in detection systems. Finally, we consider the use of small specimens to be an important asset in the study design because it ensured that the same area of the specimen was evaluated with each antibody, ensuring consistency in the comparison areas.

Our primary objective in the current study is different from the previous study. It is well-recognized that differences in the methods used to evaluate HER-2/*neu* can lead to substantial differences in conclusions regarding clinical utility. Our goal in this study is to compare FDA-approved assay methods with one another and with our own in-house immunohistochemistry assays to determine which of these is the most accurate.

Variability in sensitivity is expected because formalin-fixation and paraffin-embedding adversely effect antigenicity and immunostaining of the vast majority of protein antigens and the ability of antibodies to recognize their epitope varies from antibody to antibody. Hence, one expects that formalin-fixation and paraffin-embedding will be associated with reduced immunostaining relative to that observed in frozen tissue sections from the same specimen.[2] Many believe that the loss of antigenicity in paraffin-embedded specimens can be corrected by application of heat-induced antigen retrieval to the paraffin-embedded tissue sections such as is performed with the DAKO Herceptest. However, once again, this approach has not been validated before introduction of these reagents and methods by systematic evaluation of clinical breast cancer specimens with known amplification/overexpression levels.

In September 1998, the DAKO Herceptest was approved by the FDA to evaluate HER-2/*neu* expression for patient eligibility to receive trastuzamab treatment. The evaluation consisted of a comparison of immunostaining obtained with the Herceptest to immunostaining obtained with the "Clinical Trials Assay" (CTA) previously used by Genentech, Inc, to screen patient tumors for entry to their pivotal trastuzamab clinical trials (H0648, H0649, and H0650).[36-38] The CTA used CB11 and 4D5 mouse monoclonal antibodies with an indirect avidin-biotin technique to detect HER-2/*neu* membrane receptor protein. Immunostaining with the Herceptest was found to be in agreement with the immunostaining result obtained from the CTA assay in 79% of 548 paraffin-embedded breast cancer specimens. The Ventana Pathway CB11 immunohistochemical assay was approved by the FDA as a diagnostic assay to aid in the selection of patients likely to benefit from trastuzamab therapy in November, 2000. Approval of the CB11 Pathway assay was based on concordance analysis between the DAKO Herceptest and Pathway CB11 assays. Immunostaining with Pathway CB11 was found to be in agreement with the Herceptest in 86% of 450 paraffin-embedded breast cancer specimens. Neither of these immunohistochemical assay comparisons was performed with specimens molecularly characterized for HER-2/*neu* gene amplification or overexpression. None of the specimens used for comparison had clinical outcome information available nor were any of these women part of a trastuzamab clinical trial.

In the past, few investigators have provided data related to the screening or evaluation of HER-2/*neu* antibodies for use as immunohistochemical reagents, especially in formalin-fixed, paraffin-embedded tissue sections before using those antibodies to analyze study cohorts.[6,32,55] Consequently, the conclusions of prognostic marker evaluations and predictive marker studies performed using these uncharacterized immunohistochemical assays are open to criticism. Currently, several groups have reported immunohistochemical staining with primarily the DAKO HercepTest or the Ventana immunohistochemical assay using CB11 monoclonal antibody. Some groups have compared immunohistochemical staining with these reagents to gene amplification determined by FISH with either the Vysis assay or the Ventana assay systems.[31,54]

In our current study, FISH analyses were compared with immunohistochemical assay evaluations of a group of molecularly characterized breast cancer cases. FISH of the HER-2/*neu* gene involves hybridization of a specific DNA probe with sequences complementary to the gene to permit

assessment of the number of copies of this gene in individual tumor cell nuclei. FISH technology combines the advantages of molecular methods permitting localization of HER-2/*neu* gene copies in morphologically identified tumor cell nuclei. Immunohistochemistry involves recognition of antigenic determinants of $p185^{HER\text{-}2/neu}$ in the tumor cell membranes and permits a cell-by-cell assessment of protein expression. However, HER-2/*neu* immunohistochemistry suffers from a lack of characterization of which fixatives or fixation times are suitable for preservation of antigen immunoreactivity, lack of adequate internal immunostaining controls, and subjective assessment of membrane immunostaining. Although the range of fixatives most appropriate for FISH are also not characterized, the use of normal cells, present in each breast cancer specimen, as internal hybridization controls to confirm localization of an average of two HER-2/*neu* gene copies and two chromosome 17 centromeres per normal cell nucleus permits an assessment of the appropriateness of the results. Both FISH and immunohistochemistry are applicable to tumors of all sizes and can be performed on sections from the original specimen blocks used for diagnosis. Further, if carcinoma cells were localized rather than diffusely distributed within a tumor, amplification would be detectable by FISH but, in stroma-rich breast cancers, could be diluted below detectable limits by solid matrix blotting of DNA extracted from the tumor due to abundant noncarcinoma cellular DNA.

Because of confusion about the reliability of various HER-2/*neu* assays for entry onto clinical trials evaluating responsiveness to experimental treatments with trastuzamab and because accurate assignment of women with breast cancer for clinical management decisions is now so important, we evaluated two FISH assay methods for gene amplification and a group of HER-2/*neu* immunohistochemical assays that are either in wide-spread use for clinical testing (Ventana CB11 and DAKO HercepTest) or have been used in our own laboratory (R60 and 10H8) for assessment of HER-2/*neu* overexpression, comparing results with these assays to the results of HER-2/*neu* gene amplification/overexpression obtained using standard molecular biologic methods. Our findings demonstrated that the FISH assays have higher sensitivity and higher accuracy and more frequently correctly identify altered HER-2/*neu* status (amplification/overexpression) in previously molecularly characterized specimens than did the FDA-approved immunohistochemistry assays interpreted manually. The in-house immunohistochemical assays most frequently used in our laboratory had a sensitivity that was slightly lower but did not differ significantly from the FISH assay results. Based on these findings, if one's selection of HER-2/*neu* assays is restricted to commercially available assays, we recommend the use of FISH assays until the short-comings of tissue processing, limited antibody sensitivity for fixed antigen, and variable immunohistochemical scoring can be satisfactorily resolved. At the time when these issues are adequately resolved assay comparability should be reassessed.

Standardized thresholds for interpretation of immunohistochemical staining that can be exported from laboratory to laboratory and pathologist to pathologist are needed. Although manual interpretation of HER-2/*neu* immunostaining in our laboratory and in the laboratories of some other investigators[53] has been performed in a standardized fashion, the results obtained by others in other laboratories can be quite different. Our approach to manual or subjective interpretation of HER-2/*neu* immunostaining has been different from that of several other investigators. Few have used our in-house reagents because they have been produced in our laboratories and are not commercially available.[2,18,35] Our approach to scoring immunostaining also differs from that of other investigators. Some have used the percentage of immunostained tumor cells to score overexpression with membrane immunostaining with a certain percentage of positively stained tumor cells considered overexpression,[20,24,28,29,56,57] whereas we consider membrane intensity, not percentage of stained cells, to be the most important criterion. Our experience with frozen tissues demonstrates relatively little cell-to-cell heterogeneity within a given cancer. Cancers with amplification and overexpression have overexpression in essentially all tumor cells to a similar level.[2,18] However, formalin-fixation and paraffin-embedding of tumor tissue from breast cancers previously evaluated as frozen specimens demonstrates both a loss of immunoreactivity and the introduction of heterogeneity that was not present in the original material.[2,6]

In contrast to the experience of some investigators,[30,31] in this study we had an exceptionally low rate of false-positive results observed with heat-induced antigen retrieval performed with the DAKO HercepTest immunohistochemical assay. However our false-negative rate was significant. There were no characteristics of the immunostains (or the tumor morphology) that permitted us to predict which cases would be false-negatives with any of the immunohistochemical stains. This high false-negative rate and low false-positive rate may be related to differences in tissue processing because our tissues were processed in buffered formalin not in alcoholic formalin.[30,31] However, the problem of wide variability in interpretation of HER-2/*neu* immunostaining has become increasingly apparent. The high false-positive results observed by some investigators may not be only due to differences in tissue fixation and specimen processing, they may be related to differences in interpretation of the immunostained specimens. For example, in our clinical reference laboratory we have observed

substantial variability in the interpretation of HER-2/*neu* status for immunostained slides or tissue samples provided to obtain a second or third opinion (unpublished data). These preliminary results showed agreement of our laboratory assay results and interpretations with the immunohistochemistry assays performed and interpreted by outside pathologists in only approximately 53% of cases with regard to interpretation of HER-2/*neu* overexpression versus low expression status (2+/3+ *v* 0/1+). Similarly, a review of the first 680 cases analyzed in our centralized Breast Cancer International Research Group (BCIRG) laboratory for evaluation of HER-2/*neu* gene amplification as part of the entry criteria for BCIRG005 and BCIRG006 clinical trials demonstrated concordance with the outside or local immunohistochemistry staining result in only approximately 83% of breast cancers (unpublished data). Most of these discrepancies with outside laboratories in both our reference laboratory specimens and our BCIRG Central Laboratory specimens were false-positive results in the outside laboratory.

In addition to differences in tissue processing, assay reagents used in immunohistochemistry and differences in performing the immunohistochemical assay with or without antigen retrieval and differences in subjective interpretation of the assay can lead to substantial differences in assessment of overexpression in immunohistochemical assays. Such striking variability in interpretation of FISH results has not been demonstrated. The College of American Pathologists (CAP) reported the CAP survey results for proficiency in HER-2/*neu* testing.[58] In contrast to the results from immunohistochemistry laboratories, complete concordance was observed among the results obtained from laboratories performing FISH assays.[58] Our experience has been similar with FISH in our centralized BCIRG laboratory compared with FISH performed in referring laboratories showing a concordance rate of 96% (unpublished data).

In conclusion, FISH assays proved to be highly sensitive and specific for detecting HER-2/*neu* gene amplification. Although less sensitive, our interpretations of in-house immunohistochemical assays were not significantly different from the FISH assays for identification of overexpression. However, two commercially available, FDA-approved immunohistochemical assays were significantly less sensitive at detecting breast cancers with overexpression and were, therefore, less accurate.

ACKNOWLEDGMENT

We thank Ivonne Villalobos for assistance in the preparation of this manuscript, Craig Sawtelle from Ventana Medical Systems, Inc, for participating in CB11 immunostaining, and Karen Petrosyan, MD, for technical assistance.

REFERENCES

1. Slamon D, Clark G, Wong S, et al: Human breast cancer: Correlation of relapse and survival with amplification of the HER-2/*neu* oncogene. Science 235:177-182, 1987

2. Slamon D, Godolphin W, Jones L, et al: Studies of the HER-2/*neu* proto-oncogene in human breast and ovarian cancer. Science 244:707-712, 1989

3. Muller W, Sinn E, Pattengale P, et al: Single-step induction of mammary adenocarcinoma in transgenic mice bearing the activated c-*neu* oncogene. Cell 54:105-115, 1988

4. Chazin V, Kaleko M, Miller A, et al: Transformation mediated by the human HER-2 gene independent of the epidermal growth factor receptor. Oncogene 7:1859-1866, 1992

5. DiFiore P, Pierce J, Kraus M, et al: *erb* B-2 is a potent oncogene when overexpressed in NIH/3T3 cells. Science 237:178-182, 1987

6. Press M, Pike M, Chazin V, et al: HER-2/*neu* expression in node-negative breast cancers: Direct tissue quantitation by computerized image analysis and association of overexpression with increased risk of recurrent disease. Cancer Res 53:4960-4970, 1993

7. Press M, Bernstein L, Thomas P, et al: HER-2/*neu* gene amplification by fluorescence in situ hybridization: Evaluation of archival specimens and utility as a marker of poor prognosis in node-negative invasive breast carcinomas. J Clin Oncol 15:2894-2904, 1997

8. Gullick W, Love S, Wright C, et al: c-*erb* B-2 protein overexpression in breast cancer is a risk factor in patients with involved and uninvolved lymph nodes. Br J Cancer 63:434-438, 1991

9. Kallioniemi O-P, Holli K, Visakorpi T, et al: Association of c-*erb* B-2 oncogene overexpression with high rate of cell proliferation, increased risk for visceral metastasis and poor long-term survival in breast cancer. Int. J Cancer 49:650-655, 1991

10. Winstanley J, Cooke T, Murray G, et al: The long term prognostic significance of c-*erb* B-2 in primary breast cancer. Br J Cancer 63:447-450, 1991

11. Paterson M, Dietrich K, Danyluk J, et al: Correlation between c-*erb* B-2 amplification and risk of early relapse in node-negative breast cancer. Cancer Res 51:556-567, 1991

12. Gusterson B, Gelber R, Goldhirsch A, et al: Prognostic importance of c-*erb*B-2 expression in breast cancer: International (Ludwig) Breast Cancer Study Group. J Clin Oncol 10:1049-1056, 1992

13. Quenel N, Wafflart J, Bonichon F, et al: The prognostic value of c-*erb* B2 in primary breast carcinoma: A study on 942 cases. Breast Cancer Res Treat 35:283-291, 1995

14. Seshadri R, Firgaira F, Horsfall D, et al: Clinical significance of HER-2/*neu* oncogene amplification in primary breast cancer: The South Australian Breast Cancer Study Group. J Clin Oncol 11:1936-1942, 1993

15. Albanell J, Bellmunt J, Molina R, et al: Node-negative breast cancers with p53(-)/HER2-neu(-) status may identify women with very good prognosis. Anticancer Res 16:1027-1032, 1996

16. Ro JS, El-Naggar A, Ro JY, et al: c-*erb* B-2 amplification in node-negative breast cancer. Cancer Res 49:6941-6944, 1989

17. Andrulis I, Bull S, Blakstein M, et al: neu/erbB-2 amplification identifies a poor-prognosis group of women with node-negative breast cancer: Toronto Breast Cancer Study Group. J Clin Oncol 16:1340-1349, 1998

18. Slamon D, Press M, Godolphin W, et al: Studies of the HER-2/*neu* proto-oncogene in human breast cancer. Cancer Cells 7:371-380, 1989

19. Rilke F, Colnaghi M, Cascinelli N, et al: Prognostic significance of HER-2/*neu* expression in breast cancer and its relationship to other prognostic factors. Int J Cancer 49:44-49, 1991

20. Muss H, Thor A, Berry D, et al: *cerb* B-2 expression and response to adjuvant therapy in women with node-positive early breast cancer. N Engl J Med 330:1260-1266, 1994

21. Tetu B, Brisson J: Prognostic significance of HER-2/neu oncoprotein expression in node-positive breast cancer: The influence of the pattern of immunostaining and adjuvant therapy. Cancer 73:2359-2365, 1994

22. Tetu B, Brisson J, Plante V, et al: p53 and c-*erb* B-2 as markers of resistance to adjuvant chemjotherapy in breast cancer. Mod Pathol 11:823-830, 1998

23. Allred D, Lark G, Tandon A, et al: HER-2/*neu* in node-negative breast cancer: Prognostic significance of overexpression influenced by the presence of in situ carcinoma. J Clin Oncol 10:599-605, 1992

24. Thor A, Berry D, Budman D, et al: *erb* B-2, p53, and efficacy of adjuvant therapy in lymph node-positive breast cancer. J Natl Cancer Inst 90:1346-1360, 1998

25. Paik S, Bryant J, Park C, et al: *erb* B-2 and response to doxorubicin in patients with axillary lymph node-positive, hormone receptor-negative breast cancer. J Natl Cancer Inst 90:1361-1370, 1998

26. Wright C, Nicholson S, Angus B, et al: Relationship between c-*erb* B-2 protein product expression and response to endocrine therapy in advanced breast cancer. Br J Cancer 65:118-121, 1992

27. Borg A, Baldetorp B, Ferno M, et al: *erb* B2 amplification is associated with tamoxifen resistance in steroid-receptor positive breast cancer. Cancer Lett 81:137-144, 1994

28. Carlomagno C, Perrone F, Gallo C, et al: c-*erb* B2 overexpression decreases the benefit of adjuvant tamoxifen in early-stage breast cancer without axillary lymph node metastases. J Clin Oncol 14:2702-2708, 1996

29. Sjogren S, Inganas M, Lindgren A, et al: Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. J Clin Oncol 16:462-469, 1998

30. Roche P, Ingle J: Increased HER2 with U.S. Food and Drug Administration-approved antibody. J Clin Oncol 17:434, 1999

31. Jacobs T, Gown A, Yaziji H, et al: Specificity of HercepTest in determining HER-2/*neu* status of breast cancers using the United States Food and Drug Administration-approved scoring system. J Clin Oncol 17:1983-1987, 1999

32. Press M, Hung G, Godolphin W, et al: Sensitivity of HER-2/*neu* antibodies in archival tissue samples: Potential source of error in immunohistochemical studies of expression. Cancer Res 54:2771-2777, 1994

33. Coussens L, Yang-Feng T, Chen Y-CLE, et al: Tyrosine kinase receptor with extensive homology to EGF receptor shares chromosomal location with *neu* oncogene. Science 230:1132-1139, 1985

34. Kallioniemi O-P, Kallioniemi A, Kurisu W, et al: ERBB2 amplification in breast cancer analyzed by fluorescence *in situ* hybridization. Proc Natl Acad Sci USA 89:5321-5325, 1992

35. Park J, Yang X, Park J, et al: Assessment of novel anti-p185HER-2 monoclonal antibodies for internalization-dependent therapies. Hybridoma 18:487-495, 1999

36. Cobleigh M, Vogel C, Tripathy D, et al: Multinational study of the efficacy and safety of humanized anti-HER2 monoclonal antibody in women who have HER2-overexpressing metastatic breast cancer that has progressed after chemotherapy for metastatic disease. J Clin Oncol 17:2639-2648, 1999

37. Slamon D, Leyland-Jones B, Shak S, et al: Use of chemotherapy plus a monoclonal antibody against HER2 for metastatic breast cancer that overexpresses HER2. N Engl J Med 344:783-792, 2001

38. Vogel C, Cobleigh M, Tripathy D, et al: Efficacy and safety of trastuzumab (Herceptin) as a single agent in first-line treatment of HER2-overexpressing metastatic breast cancer. J Clin Oncol 20:719-726, 2002

39. Fleiss J: Statistical methods for rates and proportions. New York, NY, John Wiley & Sons, 1981

40. Landis J, Koch G: The measurement of observer agreement for categorical data. Biometrics 33:159-174, 1977

41. Potter H, Linklater J, Allen A, et al: Magnetic resonance imaging of articular cartilage in the knee: An evaluation with use of fast-spin-echo imaging. J bone Joint Surg 80A:1276-1284, 1998

42. Ali IU, Campbell G, Lidereau R, et al: Amplification of c-*erb* B-2 and aggressive human breast tumors? Science 240:1795-1798, 1988

43. Ali IU, Campbell G, Lidereau R, et al: Lack of evidence for the prognostic significance of c-erb B-2 amplification in human breast carcinoma. Oncogene Res 3:139-146, 1988

44. Zhou D-J, Ahuja H, Cline M: Proto-oncogene abnormalities in human breast cancer: c-*erb* B-2 amplification does not correlate with recurrence of disease. Oncogene 4:105-108, 1989

45. Clark G, McGuire W: Follow-up study of HER-2/*neu* amplification in primary breast cancer. Cancer Res 51:944-948, 1991

46. Hayes D: Tumor markers for breast cancer: Current utilities and future prospects, in Hayes D (ed): Hematology/Oncology Clinics of North America: Tumor Markers in Adult Solid Malignancies. Philadelphia, PA, Saunders, 1994, pp 485-506

47. Borg A, Tandon A, Sigurdsson H, et al: HER-2/*neu* amplification predicts poor survival in node-positive breast cancer. Cancer Res 50:4332-4337, 1990

48. Tandon A, Clark G, Chamness G, et al: HER-2/*neu* oncogene protein and prognosis in breast cancer. J Clin Oncol 7:1120-1128, 1989

49. Ravdin P: Should HER2 status be routinely measured for all breast cancer patients? Semin Oncol 26:117-123, 1999

50. Baselga J, Seidman A, Rosen P, et al: HER2 overexpression and paclitaxel sensitivity in breast cancer: Therapeutic implications. Oncol 2:43-48, 1997 (suppl)

51. Pegram M, Lipton A, Hayes D, et al: Phase II study of receptor-enhanced chemosensitivity using recombinant humanized anti-p185HER2/neu monoclonal antibody plus cisplatin in patients with HER2/neu-overexpressing metastatic breast cancer refractory to chemotherapy treatment. J Clin Oncol 16:2659-2671, 1998

52. Pegram M, Slamon D: Combination therapy with trastuzumab (Herceptin) and cisplatin for chemoresistant metastatic breast cancer: Evidence for receptor-enhanced chemosensitivity. Semin Oncol 26:89-95, 1999 (suppl 12)

53. Jacobs T, Gown A, Yaziji H, et al: HER-2/*neu* protein expression in breast cancer evaluated by immunohistochemistry: A study of interlaboratory agreement. Anat Pathol 113:251-258, 2000

54. Jacobs T, Gown A, Yaziji H, et al: Comparison of fluorescence in situ hybridization and immunohistochemistry for the evaluation of HER-2/neu in breast cancer. J Clin Oncol 17:1974-1982, 1999

55. Van de Vijver M, Peterse J, Mooi W, et al: *neu* -protein overexpression in breast cancer: Association with comedo-type ductal carcinoma in situ and limited prognostic value in stage II breast cancer. N Engl J Med 319:1239-1245, 1988

56. van Diest P, van Dam P, Henzen-Logmans S, et al: A scoring system for immunohistochemical staining: Consensus report of the task force for basic research of the EORTC-GCCG. J Clin Pathol 50:801-804, 1997

57. Nelson N: Experts debate value of HER2 testing methods. J Natl Cancer Inst 92:292-294, 2000

58. Nagle RB, Tubbs RR, Roche PC, et al: Clinical laboratory assays for HER-2/*neu* amplification and overexpression: Quality assurance, standardization, and proficiency testing. Arch Pathol Lab Med (in press)